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(54) Title: SYNTHETIC POLYNUCLEOTIDES		
(57) Abstract <p>This invention relates to synthetic polynucleotides which encode lysyl oxidase, lysyl oxidase like molecules or variants of these species. The synthetic polynucleotides of the invention permit the expression of functional lysyl oxidase, lysyl oxidase like molecules or variants of these species, typically in good yield. The invention also relates to recombinant DNA molecules containing these, synthetic polynucleotides, to cells containing them and to uses of the expression products.</p> <p>in E.coli & yeast</p>		

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"Synthetic Polynucleotides"

TECHNICAL FIELD

The present invention relates to synthetic polynucleotides encoding proteins and polypeptides selected from the group consisting of lysyl oxidases, lysyl oxidase-like proteins and variants thereof; to use of the synthetic polynucleotides to provide high yields of those proteins and polypeptides; and to uses of the proteins and polypeptides produced from the synthetic polynucleotides.

BACKGROUND ART

Lysyl oxidase is a copper dependent amine oxidase that catalyses the oxidation of amines, including but not limited to primary amines and, in particular, the amine side chain of lysine. Lysine oxidation catalysed by lysyl oxidase has been observed in the oligopeptide and polypeptide chains [REDACTED] and [REDACTED]. Lysyl oxidase activity has been observed with other amine containing substrates, such as oligopeptides where the efficiency of catalyzed oxidation is dependent on adjacent sequences (Kagan et al., 1995a) such as vicinal dicarboxylic amino acid residues (Nagan and Kagan, 1994).

With varying efficiencies, lysyl oxidases can oxidise other substrates, such as butylamine and p-hydroxybenzylamine, for example, to form butyraldehyde and p-hydroxybenzylaldehyde respectively. Non-peptide reactivity is also displayed with, for example, semi-carbazide(s) and oxidation of tyramine (Palcic et al., 1995). Lysyl oxidase activity has also been observed with other amine containing molecules including inhibitors and unnatural substrates such as trans-2-phenylcyclopropylamine (Shah et al., 1993).

The majority of the work done to date in characterising lysyl oxidase has been with respect to non-human mammalian lysyl oxidases. The level of amino

- 2 -

acid homology between lysyl oxidases from different species is of the order of 90%.

"Lysyl oxidase-like proteins" have also been identified by the analysis of nucleotide and predicted amino acid sequence alignments of DNA and protein molecules which are expressed in a particular mammalian species including humans. The level of homology between these proteins and lysyl oxidases is of the order of 75%, which is a highly significant degree of homology. Evidence indicates that these molecules may function as enzymes in the extracellular space as members of a lysyl oxidase family of molecules, and in the cell to provide lysyl oxidase activities (Kim, Y. et al. 1995).

The lysyl oxidase gene encodes a single polypeptide species and there has been no observation of lysyl oxidase mRNA splice variants or isoforms of the lysyl oxidase polypeptide *in vivo* (Boyd et al., 1995).

In the production of intra- and inter-molecular crosslinked molecules, lysine oxidation in tropoelastin is a necessary step in the formation of allysine, desmosine and isodesmosine condensation products.

Crosslinked molecules, including elastin and collagen, are significant components of fully functional connective tissue. In this regard, deficiencies in lysyl oxidase such as that found in lathyrism, lead to marked phenotypic changes that can compromise the viability of an individual.

Lysyl oxidase exists in at least two forms: an intracellular protein and the more thoroughly characterised extracellular form. In the intracellular form its roles include a ras recission function, and the encoding gene has been classified in this context as a ras-recission gene or rrg (Contente et al., 1990; Kenyon et al., 1991). In its capacity as rrg, its expression is altered in an incompletely catalogued manner, to apparently reduce the oncogenic phenotype of cells expressing aberrant ras. Lysyl oxidase levels also change during differentiation and development

(Dimaculangan et al., 1994) and in response to growth factors (Green et al, 1995). Lysyl oxidase is also a secreted protein, available in the extracellular matrix of some connective tissues in very low concentrations.

5 Difficulties in obtaining sufficient quantities of enzyme for biochemical analysis have impeded detailed exploration of its properties. Yields of purified naturally occurring lysyl oxidase available from typical purification procedures have been limited to 2-4mg
10 starting with 0.5 to 1kg of cleaned bovine aortae (Kagan and Cai, 1995).

Extracellular lysyl oxidase is typically made as a larger protein, which includes a collection of amino acid residues at the amino terminus of the protein. This
15 form, termed preprolysyl oxidase is secreted from the cell in a process contemporaneous with cleavage of the amino-terminal region to generate prolysyl oxidase which in turn is cleaved outside the cell to generate the mature form of the protein.

20 The extracellular (and possibly intracellular) lysyl oxidase enzyme additionally contains copper and at least one organic cofactor which is postulated to be a quinone-like component. The organic cofactor has variously been considered to be covalently or non-covalently bound
25 to the enzyme (Williamson et al., 1986; Kagan and Trackman, 1991). Examples of organic cofactors, which have been found to be associated with oxidases, include quinones such as P.Q.Q., topa quinone (2,4,5 trihydroxyphenylalanine quinone) and tryptophan
30 tryptophylquinone (Tanizawa, 1995). The organic cofactor for lysyl oxidase is now known to be a quinone. It is possible that the cofactor can be supplied, generated by interaction of a quinone derivative with the depleted apo-form of the enzyme, and/or generated by protein
35 oxidation such as that mediated by the participation of copper. There is at least one atom of copper tightly bound per one molecule of functional enzyme. However it was experimentally demonstrated (Gacheru et al., 1990)

that when copper is removed by a chelating agent the inactive apoenzyme can be restored to its former level of activity by the addition of copper.

Posttranslational modification(s) such as
5 glycosylation have not been described for the mature form of lysyl oxidase, although there is evidence for glycosylation of the amino terminal region destined for removal during maturation of the precursor prolysyl oxidase (Cronshaw et al., 1995). As other forms of
10 modification exist in vertebrate cells, and include participation by the cytosol, Golgi and secretion machinery, followed by extracellular processing, uncertainty has surrounded the question of whether a functional lysyl oxidase could be made in host cells
15 other than vertebrate systems.

Isolation of endogenous lysyl oxidase from, for example, mammalian tissue typically uses chemical agents which interfere with protein association, such as urea. Modest amounts of lysyl oxidase can be recovered in this
20 way from skin (Shackleton and Hulmes, 1990). The purified material is typically maintained in solutions containing chemical agent(s), in part to minimise protein aggregation and loss of catalytic function. When prepared in this way, the enzyme displays sluggish
25 activity, which has led to the assertion that the enzyme (or a macromolecular complex) (Cronshaw et al., 1993) is altered during the relatively harsh extraction procedure (Shackleton and Hulmes, 1990).

To circumvent difficulties associated with the use
30 of the purified naturally occurring enzyme, recombinant production is a logical alternative. However despite the fact that inferred lysyl oxidase sequences including human (Mariani et al., 1992; Svinarich et al., 1992; Kenyon et al., 1993), rat (Trackman et al., 1990) and
35 mouse (Contente et al., 1993) have been available for several years there have been no reports in the literature of production of recombinant functional lysyl oxidase using non-mammalian hosts for other than modest

levels of expression and no functional expression of the human sequence. Kagan et al. (1995b), have reported the production of modest levels of porcine lysyl oxidase from cDNA in mammalian cells indicating that bacterial
5 production of functional enzyme was problematic. One of the impediments to the production of recombinant lysyl oxidase has been the discrepancies appearing in some of the available sequence information (e.g. Trackman et al., 1990; Hamalainen et al., 1991 vs Mariani et al., 1992).

10 DESCRIPTION OF THE INVENTION

The paucity of information in the literature in relation to the nature of host-specific post-translational modifications of lysyl oxidase (e.g. Cronshaw et al. 1995), together with the real absence of
15 reports of functional lysyl oxidase production in non-mammalian recombinant expression systems, teaches against the choice of non-mammalian hosts for expression of nucleotide sequences encoding mammalian lysyl oxidase and implies that non-mammalian host cells are unlikely to
20 produce functional lysyl oxidase which is encoded by a mammalian nucleotide sequence.

The present inventor has reasoned that expression of mammalian lysyl oxidase in non-mammalian host cells might be achieved using a synthetic polynucleotide which
25 encodes lysyl oxidase but which has been specifically designed for use in non-mammalian host cells such as (but not limited to) *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia*. In particular, the inventor proposed the hypothesis that non-mammalian host cells
30 have difficulty expressing from nucleotide sequences encoding mammalian lysyl oxidase because of the presence of 'rare' codons in those sequences which are used infrequently in the non-mammalian host cell. Rare codons are codons that are found at low frequency in highly
35 expressed genes in a particular host. A measure of efficiencies of codon usage is the codon adaptation index (CAI) (Sharp and Li 1987). Highly expressed genes such

as those encoding ribosomal proteins in *E. coli* typically have high CAI values that are greater than or equal to 0.45, whilst poorly expressed genes have much smaller CAI values; for example, the *E. coli lacI* gene has a CAI of
5 0.3

The inventor calculated the CAI of a nucleotide sequence encoding a secreted form of human lysyl oxidase for a number of non-mammalian hosts by considering the usage of codons in the nucleotide sequence shown in
10 Figure 1. The CAI is calculated to be 0.24 for *E. coli* and 0.11 for *Saccharomyces cerevisiae*. The inventor identified codons in the nucleotide sequence encoding human lysyl oxidase which are infrequently used in host cells such as *E. coli* including: an AGGAGA cluster
15 encoding adjacent arginine residues at positions 937 to 942 which has the capacity to limit expression and generate translational pausing and frameshifting in *E. coli*; 11 other instances of single rare AGG and AGA arginine codons capable of instigating translational
20 hopping (Kane 1992); and an ensemble of codons rarely found in highly expressed *E. coli* genes (Table 1). Together these codons account for 28% of the amino acid coding capacity of the sequence.

Table 1

Codons found in the natural lysyl oxidase sequence of Figure 1 which are rarely found in highly expressed *E. coli* genes

5	Codon	Amino acid	Frequency in highly expressed genes %	Occurrence in Figure 1 residues (% total sequence)
	AGG	Arg	0.14	6 2.4
	AGA	Arg	0.21	7 2.8
	CGA	Arg	0.31	2 0.8
	CCC	Pro	0.43	7 2.8
10	TGT	Cys	0.47	7 2.8
	TGC	Cys	0.61	3 1.2
	ACA	Thr	0.65	7 2.8
	CCT	Pro	0.66	6 2.4
	TCA	Ser	0.68	3 1.2
15	GGA	Gly	0.70	4 1.6
	AGT	Ser	0.72	6 2.4
	CCA	Pro	0.82	2 0.8
	TCC	Ser	0.94	4 1.2
	GGG	Gly	0.97	2 0.8
20	CTG	Leu	0.99	6 2.4

In consideration of the CAI values for the nucleotide sequence encoding human lysyl oxidase in *E. coli* and *Saccharomyces cerevisiae*, the inventor reasoned that the problem associated with expression of

25 verterbrate lysyl oxidase or lysyl oxidase-like molecules in particular host cells or expression systems was directly related to the high proportion of codons in the verterbrate lysyl oxidase or lysyl oxidase-like protein nucleotide sequence which are rarely used by the

30 particular host cells. The inventor further reasoned that verterbrate lysyl oxidase and lysyl oxidase-like molecules might be expressed in alternative host cells if

the nucleotide sequences encoding those molecules were able to be specifically designed so as to favour higher levels of expression of lysyl oxidase in a particular alternative host cell. The inventor reasoned that

5 nucleotide sequences which encode vertebrate lysyl oxidase or lysyl oxidase-like proteins and which have been specifically designed for high level expression in a particular host may also be suitable for expression in other host cells provided that the specifically designed

10 nucleotide sequence contained codons which were frequently used by the other host cells. Moreover, the inventor realised that the concept of modifying the nucleotide sequence would find application in producing high level expression of lysyl oxidase or lysyl oxidase-

15 like proteins in the natural host cell, in particular, high level expression of nucleotide sequences encoding human lysyl oxidase and lysyl oxidase-like proteins in human cells.

The present invention provides high level expression

20 of nucleotide sequences encoding lysyl oxidases and lysyl oxidase-like proteins in recombinant expression systems. The inventor has recognised that lysyl oxidases, lysyl oxidase-like proteins and variants thereof can be used in a variety of, for instance, pharmaceutical applications,

25 but these uses require significant quantities of lysyl oxidase, lysyl oxidase-like proteins or variants thereof in a native conformation. Thus the realisation of the potential application of lysyl oxidase, lysyl oxidase-like expression products and variants thereof first

30 requires a system which allows high level production of those products in native conformation.

The benefits of being able to produce significant amounts of expression product in a functional

35 conformation include specific oxidation, rather than random oxidation, so as to produce cross-linked complexes including cross-linked elastin or cross-linked collagen which are structurally and functionally similar to normal physiological complexes. Secondly, the expression

product which exists in a functional or native conformation is unlikely to cause an adverse reaction *in vivo*, whether the expression product is directly administered *in vivo* or in the situation where residual expression product remains in a cross-linked complex after catalysis, wherein that complex is intended for *in vivo* use.

The invention provides for high level expression of vertebrate nucleotide sequences encoding lysyl oxidase and lysyl oxidase-like proteins by generating synthetic polynucleotides which encode lysyl oxidase or lysyl oxidase-like proteins. Synthetic polynucleotides are nucleotide sequences which have been specifically designed for high level expression of lysyl oxidase or lysyl oxidase-like proteins in particular host cells.

Synthetic polynucleotides of the invention are generated by introducing specific nucleotide mutations at specific positions with respect to a particular codon, so as to replace codons which are rarely used, with codons which are frequently used by the particular host. Typically the mutation will not encode an amino acid mutation. To this extent, the nucleotide mutations within the synthetic polynucleotide are silent mutations. The number of codons corresponding to each amino acid residue is shown in Table 2. As a result of the degeneracy of the genetic code it is recognised that for some amino acid residues, silent mutations may be introduced at any one of nucleotide positions 1, 2 or 3 of a particular codon. It is further recognised that for some amino acid residues, for example cysteine, arginine, glutamine, few silent mutations may be introduced. In particular methionine and tryptophan residues are both generally encoded by a single codon. Nonetheless, the present inventor anticipates that, as a result of the known low stringency codon-anti-codon interaction at nucleotide position 3 which gives rise to the "wobble effect", (Crick, F. et al. 1966) further silent mutations may be introduced at position 3, so as to increase the

number of codons for a particular amino acid residue which may be selected by a particular host cell.

The Genetic Code					
First Position (5' end)	Second Position				Third Position (3' end)
	U	C	A	G	
U	PHE	SER	TYR	CYS	U
	PHE	SER	TYR	CYS	C
	LEU	SER	Stop	Stop	A
	LEU	SER	Stop	TRP	G
C	LEU	PRO	HIS	ARG	U
	LEU	PRO	HIS	ARG	C
	LEU	PRO	GLN	ARG	A
	LEU	PRO	GLN	ARG	G
A	ILE	THR	ASN	SER	U
	ILE	THR	ASN	SER	C
	ILE	THR	LYS	ARG	A
	MET	THR	LYS	ARG	G
G	VAL	ALA	ASP	GLY	U
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	GLY	A
	VAL	ALA	GLU	GLY	G
<p>Note: Given the position of the bases in a codon, it is possible to find the corresponding amino acid. For example, the codon (5')AUG(3') on mRNA specifies methionine, whereas CAU specifies histidine. UAA, UAG and UGA are termination signals. AUG is part of the initiation signal, and it codes for internal methionines as well.</p>					

The present invention also relates to synthetic polynucleotides encoding lysyl oxidase or lysyl oxidase-like proteins wherein the nucleotide sequence of the synthetic polynucleotide comprises mutations which encode amino acid mutations. The lysyl oxidase and lysyl oxidase-like proteins which contain amino acid sequence mutations are variants of lysyl oxidase or lysyl oxidase-like proteins and are also described herein as variants of the invention. As described below, variants of the invention retain the functional properties of lysyl oxidase and lysyl oxidase-like proteins, namely that they catalyse oxidation of amine groups including lysine. The

amino acid sequence of lysyl oxidase or lysyl oxidase-like proteins may be mutated by addition, deletion, substitution or combinations thereof of the encoding nucleotide sequence and such altered molecules are
5 variants of the invention provided they retain the required activity. In particular, the inventor has recognised that the amino acid sequence of lysyl oxidase or lysyl oxidase-like proteins may be altered without significantly changing the specificity or efficacy of the
10 enzyme, so that the enzyme containing the amino acid mutation is substantially the same as the enzyme comprising the native amino acid sequence. The inventor also recognises that the specific activity and substrate specificity may be altered by mutation of lysyl oxidase
15 or lysyl oxidase-like protein amino acid sequence.

Thus the present invention further provides amino acid sequence variants of lysyl oxidase and lysyl oxidase-like proteins. Variants of the invention retain the functional properties of lysyl oxidase and lysyl
20 oxidase-like proteins, namely that they catalyse oxidation of amine groups including lysine. Variants of the invention have amino acid sequences which are homologous with the amino acid sequence of lysyl oxidase or lysyl oxidase-like proteins. For the purposes of this
25 description, "homology" between the amino acid sequence of a lysyl oxidase or lysyl oxidase-like protein and that of a variant connotes a likeness short of identity, indicative of a derivation of one sequence from the other. In particular, an amino acid sequence is
30 homologous to that of a lysyl oxidase or lysyl oxidase-like protein if the alignment of that amino acid sequence with the sequence of a lysyl oxidase or a lysyl oxidase-like protein reveals a similarity of about 65% over any
20 amino acid stretch or over any repetitive element of
35 the molecules shorter than 20 amino acids in length. Such a sequence comparison can be performed via known algorithms such as the one described by Lipman and Pearson (1985), which are readily implemented by

computer. Similarity is observed between amino acids where those amino acids have a side chain which confers a similar chemical property in the same chemical environment. For example, threonine and serine are
5 similar amino acids; aspartic acid and glutamic acid are similar amino acids; valine, leucine and isoleucine are similar amino acids etc. Thus an amino acid sequence may be considered homologous with the amino acid sequence of a lysyl oxidase or a lysyl oxidase-like protein because
10 the alignment of those sequences reveals a similarity of 65%, although at each amino acid position in the aligned sequences, none of the residues are identical.

According to a first aspect of the present invention, there is provided a synthetic polynucleotide
15 encoding an amino acid sequence selected from the group consisting of lysyl oxidases, lysyl oxidase-like proteins and variants thereof.

The lysyl oxidase may be a vertebrate including human, bovine, porcine, rat or mouse lysyl oxidase, or
20 avian lysyl oxidase including chick lysyl oxidase. Preferably, the lysyl oxidase is a human lysyl oxidase. The nucleotide sequences of rat and mouse lysyl oxidase are reported in Trackman et al. (1991), porcine in Cronshaw et al. (1993), mouse in Contente et al. (1993),
25 and chick in Wu et al. (1992).

The lysyl oxidase-like proteins of the present invention are proteins which exhibit an amine oxidation catalysing function.

The variants of lysyl oxidases and lysyl oxidase-like proteins in accordance with the present invention
30 may be truncated forms of the lysyl oxidase or lysyl oxidase-like proteins wherein the truncated forms retain the functional properties of lysyl oxidase and lysyl oxidase-like proteins, in particular the ability to
35 catalyse the oxidation of amine groups, including lysine. *In toto*, the truncated forms are typically greater than 20 amino acids in length.

The variants of the invention also include molecules that comprise a particular sequence of amino acids or a particular domain of a lysyl oxidase or lysyl oxidase-like protein wherein the sequence of amino acids or domain retain the functional properties of lysyl oxidase and lysyl oxidase-like proteins, in particular the ability to catalyse the oxidation of amine groups, including lysine.

Synthetic polynucleotides according to the invention are generated by mutating the native nucleotide sequence encoding a lysyl oxidase or lysyl oxidase-like protein or variant so that:

- a) all or some of the codons which hamper expression in the expression system in which the polynucleotide is to be expressed, are replaced with codons more favourable for expression in the expression system; and
- b) the altered sequence encodes a protein or polypeptide selected from the group consisting of lysyl oxidases, lysyl oxidase-like proteins and variants thereof.

In one preferred embodiment the synthetic polynucleotide comprises a nucleotide sequence which encodes lysyl oxidase wherein the nucleotide sequence has a CAI score of at least 0.3 in a particular host cell. More preferably the synthetic polynucleotide comprises a nucleotide sequence which encodes human lysyl oxidase, wherein the synthetic polynucleotide has a CAI of at least 0.3 in *E. coli*. Most preferably the synthetic polynucleotide comprises all or part of the nucleotide sequence shown in Figure 2 (SEQ ID NO:3), starting at residue 141 (Met) or 144 (Asp). In the sequence shown in Figure 2 (SEQ ID NO :3) the N-terminal encoded amino acid is serine. The presence of the serine residue is the consequence of the insertion of a BamHI restriction site at this position. It will be readily recognised that this serine residue could be deleted or replaced by an alternative residue related to the restriction site

present at the N-terminal end of the construct.

Similarly, at the 3' end of the construct, additional stop codons are inserted compared to the parent sequence shown in Figure 1 (SEQ ID NO: 1). These stop codons are
5 a feature of this particular construct.

With respect to the sequence of the lysyl oxidase molecule, the first 21 amino acids of the sequence shown in Figure 1 (SEQ ID NO:2) are amino acids of the pre-sequence. The residues from this position and up to and
10 including residue 143 comprise the pro-sequence. Thus the mature form of the lysyl oxidase commences at residue 144. These different constructs are encompassed within the synthetic polynucleotides and encoded products of the invention.

15 In another preferred embodiment, at least 50% of codons in a particular nucleotide sequence which may be altered so as to reflect frequent codon usage in a particular host, are selected and altered. More preferably at least 60% are altered. Most preferably at
20 least 70% are altered.

In another preferred embodiment at least 50% of codons which encode a particular amino acid are altered, so as to reflect frequent codon usage in a particular host. More preferably at least 60% are altered. Most
25 preferably at least 70% are altered.

Modifications to codon usage to provide enhanced expression for a variety of hosts are discussed in: Zhang et al. (1991), for *E. coli*, yeast, fruit fly and primates where codon usage tables are provided;
30 Newgard et al. (1986), for mammals; and Murray et al. (1989), for plants. Preferred codon usages are indicated in these publications.

It is important to recognise that even where human originating nucleotide sequences are to be expressed in
35 human cell lines, or other mammalian cell lines, that modifications to codon usage can still be beneficial to producing enhanced levels of the protein of interest. The same applies to expression of other lysyl oxidase and

lysyl oxidase like proteins in cell lines derived from their originating species or other mammalian or avian species.

5 In another preferred embodiment, all or part of the 5' or 3' untranslated region, or intronic regions of the synthetic nucleotide sequence encoding the lysyl oxidase or lysyl oxidase-like protein or variant thereof, are deleted.

10 In another preferred embodiment, all or part of the regions encoding the signal peptide, or regions encoding the pro-peptide, of the synthetic nucleotide sequence encoding the lysyl oxidase or lysyl oxidase-like protein or variant thereof, are deleted.

15 More preferably, all or part, of the 5' or 3' untranslated region, and/or intronic regions, and regions encoding the signal peptide, and/or regions encoding the pro-peptide, of the synthetic polynucleotide encoding the lysyl oxidase or lysyl oxidase-like protein or variant thereof are deleted.

20 It will be recognised that the strategies for specific synthetic gene construction to yield enhanced expression of lysyl oxidases described in detail herein, can readily be adapted to the synthesis of other lysyl oxidases, lysyl oxidase-like proteins and variants thereof not specifically exemplified here, in light of the significant degree of homology between these molecules and in light of the fact that the present inventor has established that these proteins can be expressed in functional form in significant amounts from particular host cells using synthetic gene constructs with enhanced codon usage.

30 In another preferred embodiment the synthetic polynucleotide comprises a nucleotide sequence encoding lysyl oxidase, lysyl oxidase-like protein or a variant thereof and a further nucleotide sequence, wherein the nucleotide sequence encoding lysyl oxidase, lysyl oxidase-like protein or a variant thereof and the further nucleotide sequence are chemically linked so as to encode

a single open reading frame. The further nucleotide sequence is preferably chemically linked to the 5' end of the nucleotide sequence encoding lysyl oxidase, lysyl oxidase-like protein or a variant thereof.

5 In another preferred embodiment, the further nucleotide sequence encodes all or part of a polypeptide which causes the lysyl oxidase, lysyl oxidase-like protein or variant thereof to be either secreted to the extracellular environment, expressed as a cell surface
10 periplasmic protein, or expressed in the intracellular environment. Preferably the further nucleotide sequence encodes all or part of glutathione S-transferase.

In another preferred embodiment the further nucleotide sequence encodes additional residues such as
15 an N-terminal methionine or formyl-methionine.

In a preferred form of the invention the synthetic polynucleotide is unfused.

In another preferred embodiment, the synthetic polynucleotide comprises a fragment of the nucleotide
20 sequence shown in Figure 2, wherein the fragment of the nucleotide sequence encodes a protein which retains the enzymatic activity of lysyl oxidase, lysyl oxidase-like protein or a variant thereof. Preferably the synthetic polynucleotide comprises the sequence shown in Figure 2
25 and commencing at the codon encoding residue 141. In another preferred embodiment the synthetic polynucleotide comprises the sequence shown in Figure 2 and commences at or near the codon encoding residue 144.

In a further preferred embodiment, the synthetic
30 polynucleotide comprises the nucleotide sequence shown in Figure 2 (SEQ ID NO:3), or a fragment thereof, in which only some of the nucleotide mutations have been introduced. Preferably, the synthetic polynucleotide comprises the nucleotide sequence shown in Figure 2 (SEQ
35 ID NO:3), or a fragment thereof, and at least 50% of the nucleotide mutations have been introduced. More preferably, the synthetic polynucleotide comprises the nucleotide sequence shown in Figure 2 (SEQ ID NO:3) in

which the number of nucleotide mutations which have been introduced produce a CAI score for the synthetic polynucleotide in *E. coli* of at least 0.30.

5 The production of the synthetic polynucleotides of the invention, which is described in detail in the accompanying Examples, can be via the techniques of organic synthesis or recombinant DNA technology, or a combination of both techniques.

10 The synthetic polynucleotides described in the accompanying Examples were designed to make them amenable to cloning and expression in fused and unfused forms, and for intracellular and secreted production.

15 Where the synthetic polynucleotide is prepared from assembled oligonucleotides it is preferred to incorporate restriction sites in the sequence to facilitate assembly of the polynucleotide.

Restriction sites incorporated in the polynucleotide sequence are also useful for:

- 20 1. facilitating subcloning of manageable blocks for sequence confirmation;
2. providing sites for later introduction of modifications to the polynucleotide as insertions, deletions or base changes;
- 25 3. facilitating confirmation of correct polynucleotide assembly by restriction endonuclease digestion.

30 The synthetic polynucleotides encoding the variants of the invention can be produced by the techniques of site-directed mutagenesis or random mutagenesis. These techniques allow one to determine the effect of mutation at particular positions in the synthetic polynucleotide sequence on the amine oxidation catalysing properties of the encoded variant of the invention.

35 In one preferred embodiment, the variants of the invention are produced by the technique of site-directed mutagenesis using oligonucleotides, which comprises the following steps:

1. synthesis of an oligonucleotide with a sequence that contains the desired nucleotide mutation;

2. hybridizing the oligonucleotide to a template nucleotide sequence encoding lysyl oxidase or a lysyl
5 oxidase-like protein; and

3. using a DNA polymerase to extend from the 3' end of the oligonucleotide in the 5' to 3' direction.

Another approach which is particularly suited to situations where the synthetic polynucleotide has been
10 prepared from oligonucleotide blocks bounded by restriction sites is cassette mutagenesis where entire restriction fragments are inserted, deleted or replaced.

According to a second aspect of the present invention there is provided a recombinant nucleic acid
15 molecule comprising a synthetic polynucleotide of the first aspect. Preferably the recombinant nucleic acid molecule comprises vector nucleic acid.

The vectors of the invention include plasmids, phages, and phagemids. The vectors of the invention can
20 be used in integrative expression systems or lytic or comparable expression systems.

Suitable vectors will generally contain origins of replication and control sequences which are derived from species compatible with the intended expression host.
25 Typically these vectors include a promoter located upstream from the synthetic polynucleotide, together with a ribosome binding site if intended for prokaryotic expression, and a phenotypic selection gene such as one conferring antibiotic resistance or supplying an
30 auxotrophic requirement. For production vectors, vectors which provide for enhanced stability through partitioning may be chosen. Where integrative vectors are used it is not necessary for the vector to have an origin of replication. Lytic and other comparable expression
35 systems do not need to have those functions required for maintenance of vectors in hosts.

For *E. coli* typical vectors include pBR322, pBluescript II SK⁺, pGEX-2T, pTrc99A, pET series vectors,

particularly pET3d, (Studier et al. 1990) and derivatives of these vectors.

5 In one preferred embodiment, the vector comprises a synthetic polynucleotide of the first aspect, wherein the synthetic polynucleotide has a CAI score of at least 0.30 in a particular host cell. Preferably the vector comprises all or part of a synthetic polynucleotide sequence shown in Figure 2 (SEQ ID NO:3).

10 In another preferred embodiment, the vector comprises a synthetic polynucleotide of the first aspect wherein at least 50% of codons in a particular nucleotide sequence which may be altered so as to reflect frequent codon usage in a particular host, are selected and altered. More preferably at least 60% are altered. Most
15 preferably at least 70% are altered.

In another preferred embodiment the vector comprises a synthetic polynucleotide of the first aspect wherein at least 50% of codons which encode a particular amino acid are altered, so as to reflect frequent codon usage in a
20 particular host. More preferably at least 60% are altered. Most preferably at least 70% are altered.

In another preferred embodiment the vector comprises a synthetic polynucleotide of the first aspect wherein all or part of the 5' or 3' untranslated region, and/or
25 intronic regions is deleted.

In another preferred embodiment the vector comprises a synthetic polynucleotide of the first aspect wherein all or part of the regions encoding the signal peptide, and/or regions encoding the pro-peptide is deleted.

30 In another preferred embodiment the vector comprises a synthetic polynucleotide of the first aspect wherein all or part, of the 5' or 3' untranslated region, and/or intronic regions, and/or all or part of the regions encoding the signal peptide, and or regions encoding the
35 pro-peptide is deleted.

In another preferred embodiment, the recombinant DNA molecule comprises a synthetic polynucleotide of the first aspect, wherein the synthetic polynucleotide

sequence is chemically linked to a further nucleotide sequence so as to encode a single open reading frame for the synthetic polynucleotide and the further nucleotide sequence. Preferably, the recombinant DNA molecule
5 comprises a synthetic polynucleotide and the further nucleotide sequence wherein the further nucleotide sequence is chemically linked to the 5' end of the synthetic polynucleotide.

In another preferred embodiment, the recombinant DNA
10 molecule comprises a synthetic polynucleotide, wherein the synthetic polynucleotide comprises all or part of the nucleotide sequence shown in Figure 2 (SEQ ID NO:2). Preferably, the recombinant DNA molecule comprises a synthetic polynucleotide sequence having all or part of
15 the sequence shown in Figure 2 (SEQ ID NO:3) wherein only some of the indicated nucleotide mutations have been introduced.

According to a third aspect of the present invention there is provided a host cell transformed with a
20 recombinant DNA molecule of the second aspect of the invention.

The host cells may be prokaryotic cells including bacteria, and may also include yeasts, insect cells and other eukaryotic cells or whole organisms.

25 In a preferred embodiment, the host is an *E. coli* strain including *E. coli* B strain derivatives (Studier et al. 1990), and *E. coli* K12 derivatives such as NM522 (Gough and Murray, 1983) and XL1-Blue (Bullock et al. 1987).

30 In another preferred embodiment, the host is *S. cerevisiae* or *Pichia*.

According to a fourth aspect of the present invention there is provided an expression product, wherein the expression product is derived from a host
35 cell of the third aspect, the expression product comprising an amino acid sequence selected from the group consisting of lysyl oxidase, lysyl oxidase-like proteins and variants thereof.

In one preferred embodiment, the expression product comprises the amino acid sequence shown in Figure 2 (SEQ ID NO:4) and is encoded by a synthetic polynucleotide which comprises the nucleotide sequence shown in (SEQ ID NO:3).

In an alternative embodiment, the expression product comprises a fragment of the amino acid sequence shown in Figure 2 (SEQ ID NO:4) which retains amine oxidation catalysing properties, and is encoded by a synthetic polynucleotide comprising the corresponding nucleotide sequence shown in Figure 2 (SEQ ID NO:3). Preferably the expression product comprises an amino acid sequence having an N-terminus at amino acid residue position 141 (Met) of Figure 2 (SEQ ID NO:4) or results in an N-terminal amino acid residue position 144 (Asp) of Figure 2, (SEQ ID NO:4) and is encoded by a synthetic polynucleotide comprising the corresponding nucleotide sequence shown in Figure 2 (SEQ ID NO:3).

In another preferred embodiment, the expression product is encoded by a synthetic polynucleotide comprising the nucleotide sequence shown in Figure 2 (SEQ ID NO : 3) in which only some of the nucleotide mutations have been introduced. Preferably the expression product is encoded by a synthetic polynucleotide comprising all or part of the nucleotide sequence shown in Figure 2 (SEQ ID NO:3) and at least 50% of the nucleotide mutations relative to Figure 1 have been introduced. More preferably the expression product is encoded by a synthetic polynucleotide comprising all or part of the nucleotide sequence shown in Figure 2 (SEQ ID NO:3) in which the number of nucleotide mutations which have been introduced produce a CAI score for the synthetic polynucleotide in *E. coli* of at least 0.30.

The expression products of the fourth aspect may be fused expression products which include all or part of a protein encoded by the vector in peptide linkage with the expression product. They may also include, for example, an N-terminal methionine or other additional residues

which do not permanently impair the catalytic properties of the product.

In a further preferred embodiment, the expression products of the invention are fusion products which
5 comprise an amino acid sequence selected from the group consisting of lysyl oxidase, lysyl oxidase-like proteins and variants thereof which are encoded by a synthetic polynucleotide, wherein the amino acid sequence is chemically linked to a further amino acid sequence.
10 Typically, the further amino acid sequence is chemically linked to the N-terminus of the expression product. Preferably the further amino acid sequence allows purification of the fusion product. Most preferably the further amino acid sequence comprises an amino acid
15 sequence encoding glutathione *S*-transferase. The further amino acid sequence may be chosen in order to cause the fusion product to be secreted to the extracellular environment, expressed as a cell membrane protein or expressed in the intracellular environment.
20 The expressed fusion products may subsequently be treated to remove the further amino acid sequence from the amino acid sequence selected from the group consisting of lysyl oxidase, lysyl oxidase-like proteins and variants thereof. Preferably, the treatment is via
25 chemical cleavage. In another more preferred embodiment, the treatment is via protease digestion. Still more preferably, the treatment is effected by endogenous host cell secretion machinery, for example yeast cell secretion machinery.

30 Non-fused systems include the introduction of or use of a pre-existing methionine codon. An example of this is the use of pET3a and pET3d in *E. coli*.

In a further embodiment, the invention provides a pharmaceutical composition comprising an expression
35 product of the invention and a pharmaceutically acceptable carrier, excipient or diluent. The pharmaceutical compositions are prepared and tested according to standard pharmaceutical practise. The

support for their efficacy is provided by the assays in the Examples.

According to a fifth aspect of the invention there is provided a method for producing an expression product of the invention, the method comprising the following steps:

(i) providing a host cell of the third aspect of the invention.

(ii) maintaining the host cell in conditions suitable for expression of the synthetic polynucleotide; and

(iii) isolating the expression product.

In one preferred embodiment the expression product is produced by expression of the synthetic polynucleotide in *E. coli*. The invention extends to the use of other host cells which are capable of producing the expression product by expression of the synthetic polynucleotides designed for use in *E. coli*.

In another preferred embodiment, the expression product is produced by expression of a synthetic polynucleotide of the invention in other microbial expression systems including prokaryotic expression systems such as bacterial expression systems, and also including insect cell expression systems, and expression systems involving other eukaryotic cells or whole organisms. Again synthetic polynucleotides which are designed for use in a particular host can also be used in other cells which provide high expression of that sequence.

As disclosed in the Examples, the inventor has determined that it is useful to add Cu to the expression product which has been purified from the inclusion bodies of *E. coli* or similar organisms, so that the expression product can fold into a functional conformation which has the enzymatic activity of lysyl oxidase or lysyl oxidase-like proteins. The role of Cu in solubilising lysyl oxidase protein was not apparent until the present invention was carried out. Indeed based on earlier reported results which show that inactive apoenzyme can

be reactivated by the addition of Cu it would have been expected that re-folding could occur without Cu. The present inventor initially attempted to refold protein in the absence of Cu and failed to generate soluble
5 protein. It was only in subsequent experiments where Cu was added that refolding in this environment succeeded.

Further, should over expressed lysyl oxidase lack copper after its production in bacteria, the incorporation of copper into the recombinant protein
10 would be possible by adapting the previously reported protocol (Gacheru et al. 1990) used for conversion of the apoenzyme of natural lysyl oxidase into the metal containing form.

In addition, it would be desirable to add oxidising
15 substances that assist in the production of the organic cofactor if this is not formed by the enzyme under the prevailing conditions without assistance. The most important additional substance is oxygen in solution.

It is proposed here that generation of the active
20 enzyme *in vitro* is a time-dependent process involving the introduction of copper to the overexpressed polypeptide, followed by oxidation of the protein to generate the covalently bound quinone. This is despite the absence of a Asn-Tyr-Asp/Glu consensus sequence from lysyl oxidase
25 presumed to be important to this type of process (Tanizawa 1995).

The expression product may be exposed to chemical agents to enhance the stability and activity of the molecule. The chemical agents include various
30 concentrations of urea or glycerol combined with storage at -20°C.

In one preferred form the expression product is produced in the form of inclusion bodies which are harvested from the transformed host.

35 As described above, the inventor recognised a variety of uses for lysyl oxidase, lysyl oxidase-like proteins and variants thereof although these uses require significant amounts of protein in functional

conformation. As the inventor has for the first time produce significant amounts of enzyme in functional conformation, the following uses, as described below can now be realised.

5 The invention provides a method of cross-linking molecules that contain primary amines, wherein the molecules are associated with a cell membrane or extracellular environment, comprising contacting the primary amine with an expression product of the
10 invention.

As described in the Examples, the inventor now recognises that the expression products of the invention have specific activity for primary amine groups in peptide and non-peptide substrates. Furthermore, as
15 described in the Examples, as the present inventor has, for the first time, observed cross-linking of both peptide and non-peptide substrates *in vitro*, using the expression product of the invention, the inventor envisages that the expression products of the invention
20 will be useful for cross-linking a wide range of molecules such as peptides and polypeptides, non-peptide chemical polymers which contain primary amine groups and combinations thereof. In particular, the present inventor envisages that the expression product of the
25 invention will be widely applicable to methods of treatment *in vivo*, in particular where those methods of treatment require cross-linking of elastin or collagen. For this purpose the expression product can be formulated in suitable carriers, buffers and other conventional
30 delivery systems.

In one preferred embodiment, the invention provides a method of cross-linking molecules that contain primary amines, wherein the molecule is a peptide or polypeptide molecule and the primary amine is associated with an
35 amino acid side chain. The method comprises contacting the peptide or polypeptide molecule with an expression product of the invention under conditions suitable to cross-link the molecules. Preferably, the peptide or

polypeptide comprises the amino acid sequence of tropoelastin or a fragment thereof. In another preferred embodiment, the peptide or polypeptide comprises the amino acid sequence of collagen or a fragment thereof.

5 It can also comprise both.

In another preferred embodiment the invention provides a method of cross-linking molecules that contain primary amines, wherein the molecule is a non-peptide molecule. The method comprises contacting the non-peptide molecule with an expression product of the invention under conditions suitable to cross-link the molecules.

The present inventor also recognises that the expression products of the invention would find wide application in accelerating the rate of wound healing. In particular the inventor has recognised that lysyl oxidase, lysyl oxidase-like proteins and variants thereof may be used to accelerate wound healing. Wound healing may be accelerated by administering an expression product of the invention to the wound. Alternatively, an expression product of the invention may be included in a matrix including an elastin, tropoelastin and/or collagen based or amine containing matrix which is itself applied to the wound. In both methods, wound healing is accelerated by cross-linking of molecules which contain primary amines, and the cross-linking is mediated by the expression product of the invention. For this purpose the expression product can be formulated in suitable carriers, buffers and other conventional delivery systems.

Thus the invention provides a method of accelerating the rate of wound healing, the method comprising the step of administering an expression product of the invention to a wound under conditions which are suitable for cross-linking of molecules containing primary amines. Preferably, an expression product of the invention is administered to the wound by direct application of the product to the wound. More preferably the product is

administered to the wound by first including the product in a matrix which is itself administered to the wound. Most preferably, the product is administered to the wound by application of a pharmaceutical formulation comprising
5 the expression product of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

In another preferred embodiment, the invention relates to a method of decelerating the rate of wound
10 healing. The inventor recognises that there are particular surgical procedures which require a delay in wound healing so as to achieve a desirable outcome. A lysyl oxidase-mediated delay in wound healing is based on the ability to inhibit the oxidation of primary amines
15 and therefore the ability to inhibit the cross-linking of particular molecules, where that cross-linking is essential to wound healing. Lysyl oxidase inhibitors are known, for example BAPN, although these reagents are typically noxious and therefor unsuitable for use in
20 methods of treatment. The inventor envisages that a delay in wound healing which is mediated by the inhibition of oxidation of primary amines can be achieved by developing novel reagents which have specific anti-lysyl oxidase activities and which have no side-effects.
25 Particularly suitable inhibitors of lysyl oxidase activities are anti-lysyl oxidase antibodies. These immunological reagents may be generated by raising antibodies to the expression products of the invention. The methods for the production of monoclonal antibodies
30 or polyclonal antibodies are within the knowledge of the skilled addressee and are described in detail in standard immunological texts. Furthermore, the skilled addressee can readily determine those antibodies with anti-lysyl oxidase activities, in particular the ability to inhibit
35 the oxidation of primary amines by performing screening of those antibodies according to the benzylamine substrate assay, tropoelastin substrate assay or

tritiated tropoelastin substrate assay, as described in the accompanying Examples.

Thus, the invention provides a method of decelerating the rate of wound healing, the method comprising the step of administering antibodies which are raised against the expression product of the invention, to a wound under conditions which allow the antibodies to substantially inhibit the oxidation of primary amines.

In another aspect embodiment, the invention relates to novel reagents for modifying the rate of wound healing. In particular, the inventor recognises that the expression products of the invention can be used to link chemical species to heterologous surfaces to substantially promote or substantially inhibit the rate of wound healing. The chemical species of interest generally include those species which contain a primary amine and which can be linked by oxidation of the primary amine to a heterologous surface, but more specifically comprise those chemical species selected from the group consisting of immunomodulatory substances including growth factors and cytokines, and substances which modify inflammatory responses or the coagulation cascade and also antibiotic substances. The heterologous surface may be a matrix comprising a complex of biomolecules, in particular elastin or tropoelastin, or a complex of non-peptide molecules. The expression product of the invention may be used to form a linkage between two chemical species which contain primary amine groups. Thus the invention has particular application to the attachment of an active ingredient to a carrier molecule.

Thus the invention provides novel reagents and formulations thereof for use in modifying the rate of wound healing, wherein the reagents are generated by oxidising a primary amine contained in a chemical species so as to link the chemical species to a heterologous surface.

In another preferred embodiment, the invention relates to the use of an expression product of the

invention in the treatment or prevention of abnormal fibrosis. Lysyl oxidase has an important functional role in fibrosis and is also believed to be involved in the abnormal process of fibrosis, in particular in the fibrotic liver (Kagan H. M. 1994). The present inventor envisages the use of the expression products of the invention in promoting fibrosis so as to achieve a normal physiological outcome and inhibiting fibrosis where an abnormal outcome is to be avoided. Fibrosis may be promoted by increasing the levels of available lysyl oxidase or by increasing the specific activity of the enzyme. For example fibrosis may be promoted by administering an expression product of the invention to a particular site so as to promote fibrosis at that site. Fibrosis may also be promoted by administering anti-lysyl oxidase antibodies which augment or enhance the specific activity of the enzyme, for example by interfering with aggregation of the protein. Fibrosis may be inhibited by inhibiting or reducing the ability of the lysyl oxidase to oxidise primary amine residues. The inventor envisages that fibrosis, as mediated through the role of lysyl oxidase in the oxidation of primary amines, could be inhibited by the use of antibodies which inhibit the oxidation reaction. As described above, the methods for the production of monoclonal antibodies or polyclonal antibodies are within the knowledge of the skilled addressee. Furthermore, the skilled addressee can readily determine those antibodies with anti-lysyl oxidase activities which either inhibit or enhance lysyl oxidase activity, in particular the ability to inhibit or enhance the oxidation of primary amines by performing screening assays according to the benzylamine substrate assay, tropoelastin substrate assay or tritiated tropoelastin substrate assay, as described in the accompanying Examples.

As described above, three separate assays are disclosed in the accompanying Examples for measuring levels of lysyl oxidase activity. The inventor

recognises that the expression products of the invention could be used in a particular method so as to screen agents for the ability to inhibit or enhance lysyl oxidase activity. Agents which are identified as having
5 inhibitory or enhancing effects on the specific activity of lysyl oxidase would be desirable for use in specific treatments, including wound healing or fibrosis, as described above.

Thus in a preferred embodiment, the invention
10 provides a method for screening agents which substantially enhance or substantially inhibit the specific activity of lysyl oxidase or lysyl oxidase-like proteins, the method comprising the step of contacting the agent with an expression product of the invention and
15 measuring the specific activity of the expression product.

To the extent that lysyl oxidase and lysyl oxidase-like proteins are involved in the oxidation of primary amines, the inventor recognises that the expression
20 products of the invention are useful in assays for detecting substrates which contain primary amines. The expression products of the invention are also useful to quantify amounts of protein or non-protein substrates wherein those substrates contain a known number of
25 primary amine moieties. The expression products of the invention are particularly suited to such an assay as the expression product is derived from a pure source and will therefore specifically detect primary amine groups. Furthermore, as the expression products of the invention
30 may be variants of lysyl oxidase or a lysyl oxidase-like protein, the inventor recognises that molecules associated with a particular primary amine (for example a primary amine group displayed in a specific manner) may be specifically detected over other molecules in the
35 sample which contain a primary amine group. The assay could be performed according to any of the three substrate assays detailed in the accompanying Examples.

Thus in a preferred embodiment, the invention relates to a method for detecting a protein or non-protein substrate in a sample, the method comprising contacting the sample with an expression product of the invention and detecting oxidation of any primary amine moieties in the sample.

In another preferred embodiment, the invention relates to a method for quantifying the amount of a protein or non-protein substrate in a sample, wherein the number of primary amine moieties per substrate molecule is known, the method comprising contacting the sample with an expression product of the invention and detecting the amount of oxidation of any primary amine moieties in the sample.

In one aspect, the lysyl oxidase, lysyl oxidase-like proteins and variants thereof may be used to cross-link a primary amine containing molecule such as tropoelastin or collagen to the surface, wherein the solid surface has primary amine groups which are available for cross-linking. In this aspect, the invention provides the use of lysyl oxidase, lysyl oxidase-like proteins and variants thereof as an agents for forming coatings on solid surfaces. The present inventor envisages that those surfaces which have primary amine groups available for cross-linking will be useful for the manufacture of synthetic tissues for example, blood vessels.

In one embodiment, it is envisaged that surface or devices could be dipped into solutions of tropoelastin and/or collagen in the presence of a lysyl oxidase, lysyl oxidase-like protein or variant of the invention.

In another embodiment, a cross-linked material could be preformed and then cross-linked to available primary amine groups on a surface, using a lysyl oxidase, lysyl oxidase-like protein or variant of the invention.

The cross-linked molecule may comprise collagen and/or tropoelastin and/or other cross-linkable primary amine containing molecules.

In another aspect, the invention provides a tissue glue, which is of use in assisting wound healing. This glue can be used as a replacement for sutures. The glue comprises an expression product of the invention and may additionally comprise at least one cross-linkable primary amine material, for example, tropoelastin and/or collagen. The glue may also be used to repair incisions in for instance, surgical applications and other body tissue defects. The glue may be applied by mixing the primary amine containing material and expression product and applying to the site.

Other uses for the expression products of the invention include the preparation of sheets or films where the product can be used as a reinforcement as well as for its biological properties. Applications could include repair of anastomoses, repair of defects in the heart. The expression products can also be used to prepare solid structures such as beads which may find application in tissue bulking applications such as in the urethra, oesophagus or in dermatological applications where primary amine containing substances are desirably deposited in cross-linked form.

The expression products of the invention are also useful as agents for:

The routes envisaged for the administration of this form of cancer therapy include gene and protein therapies in which the gene or protein is administered in accordance with protocols already developed with respect to other molecules proposed for use in gene or protein therapy. In one application category it is proposed that extracellular application of lysyl oxidase either alone or as part of a delivered complex, could reduce the growth and behaviour of cancer and other cells, including but not limited to ras-transformed cells. Lysyl oxidase administration has the potential to display the anti-cancer phenotypic

potential of drugs such as azatyrosine (Krzyszosiak et al., 1992; Contente et al., 1990).

- Regulating cell growth. Increased lysyl oxidase may be considered to mimic some of the functions mediated by transforming-growth-factor-beta 1 (Shibanuma et al., 1993).

- Glue to join primary amine materials e.g. in surgical or veterinary applications. The expression product can be formulated in suitable carriers, buffers and other conventional delivery systems. This includes natural materials, synthetic materials and composites.

- Oxidising accessible lysines (and other amines) to modify behaviour of those molecular components.

- Modified lysyl oxidase to perform further reactions.

15 BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS

Figure 1: Entire coding sequence (SEQ ID NO:1) for an amino acid sequence (SEQ ID NO:2) of a secreted form of human lysyl oxidase. A potential signal sequence (1 to 21 aa) is underlined (Mariani et al., 1992; Hamainen et al., 1991; Svinarich et al. 1992).

Figure 2: DNA sequence (SEQ ID NO: 3) of a synthetic polynucleotide SHLOX encoding the amino acid sequence (SEQ ID NO: 4) of a lysyl oxidase. Restriction sites at the boundaries of each block are identified;

Figure 3: Comparison of the synthetic sequence SHLOX with the natural sequence (GenBank HUMLOX coordinates). HUMLOX is on the upper line and SHLOX on the lower line, in each case;

Figure 4: Sequences of synthetic oligonucleotides for SHLOX construction (5' is at the left of each sequence, and 'p' denotes phosphate). (SEQ ID NOS:5-30)

Figure 5: Measure of lysyl oxidase activity as indicated by the production of tritiated water (TCA precipitated counts).

Figure 6: Measure of lysyl oxidase activity as indicated by the production of tritiated water.

BEST METHOD OF CARRYING OUT THE INVENTION

Design of a synthetic lysyl oxidase gene

Human lysyl oxidase is typically made in human cells as a longer form, which is proteolytically processed to generate a shorter protein secreted into the extracellular space. The amino acid sequence has been compiled from data from different sources: complete sequence data which was available for cDNA encoding human lysyl oxidase (Hamalainen et al., 1991; Mariani et al., 1992), as well as partial human genome sequence (Svinarich et al., 1992). Analytical comparison of the cDNA sequences showed a discrepancy in amino acid composition which required elucidation before the synthetic gene could be made, namely Ala136(GCT) was in conflict with Arg136(CGT) of Hamalainen et al., (1991). The present inventor dealt with this by considering the genomic data, and analysing homologous regions of lysyl oxidase from different species, including rat, mouse, bovine and chicken. The choice made was later confirmed (Hamalainen et al., 1993) supporting the correction that had been made in generating the synthetic gene. The synthetic gene was designed with strategically placed restriction sites to facilitate conversion to novel derivatives and counterparts of lysyl oxidase and lysyl oxidase-related forms (Kim et al., 1995). The synthetic gene was designed with a codon usage pattern favourable to high level expression in *E. coli* (Figure 2).

As lysyl oxidase is a secreted protein its cDNA derived sequence encodes a region for a signal peptide, which would not be useful for generating a functional enzyme in *E. coli* but rather for directing eventual transfer of the newly translated protein from inside the cell into the extracellular matrix. Furthermore the secreted pro-lysyl oxidase was thought to be cleaved by extracellular protease(s) to process the mature protein, yet it was not known if the pre-cleaved protein was active. There were doubts regarding the precise

boundaries of the presumably removed signal peptide and propeptide ("leading peptide"). The likely leading peptide sequence in the N-terminal part of the lysyl oxidase sequence was determined by computer analysis and from the definitions provided by others (Trackman et al., 1990; Hamalainen et al., 1991) and was excluded from gene synthesis. Subsequent to this, further modifications were made to adjust for varied N-terminal sequences, various candidate signal sequences and eventually a better estimate of the leading peptide sequence, to generate derivatives of SHLOX, as detailed below.

Initial gene construction and cloning was conducted as described by Martin et al. (1995). The lysyl oxidase sequence of Figure 1 was backtranslated according to a table of average codon frequencies for a group of highly expressed *E. coli* genes (GCG package version 7-UNIX using CodonFrequency and GenRunData: ecohighcod). The resulting codon sequence lacked rare codons. Modifications were made to the refined sequence to allow for convenient construction, cloning and analysis by adding short artificial sequences to each end of the codon-optimized gene to provide *Bam*HI-compatible cohesive termini, an *Nco*I site near the 5' end of the gene, and three stop codons at the end of the open reading frame. Restriction sites were introduced into the synthetic coding sequence at 270 - 300 base pair intervals, dividing the gene into 4 sections of a manageable size for individual assembly, cloning and sequence analysis.

Each section was subdivided into pairs of complementary oligonucleotides. Internal junctions between oligonucleotide pairs were delineated by nucleotide overlaps, whilst the external junctions fall at the restriction enzyme cleavage points. The codon-optimized sequence and restriction sites are shown in Figure 2, together with the encoded amino acid sequence. Oligonucleotide sequences and designations are shown in Figure 4.

Oligonucleotide pairs 2 to 6 were made by the Sydney University Macromolecular Analysis Centre, Australia and subsequently purified by denaturing polyacrylamide gel electrophoresis (PAGE) as follows. Oligonucleotides

5 (>85-mers) were run on PAGE containing 7M urea and 8% to 10% acrylamide (acrylamide:bisacrylamide 19:1). Samples were heated to 75°C for 3 minutes before loading. Tracking dye (0.05% bromphenol blue, 0.05% xylene cyanole FF in deionized formamide) was loaded into an adjacent

10 lane and electrophoresis conducted at constant power of 25 W (ca. 300 V) until the bromphenol marker was within 1cm of the base of the gel. Product bands were visualized by UV-shadowing over a fluorescent TLC plate. Gel fragments containing purified oligonucleotides were

15 excised and transferred to microcentrifuge tubes, crushed and soaked overnight at 60°C in 500µl elution buffer (0.3M sodium acetate pH7.0). A second extraction was performed with 400µl elution buffer, for 3 to 4 hours at 60°C and the supernatant combined with that of the first

20 extraction. The total volume of the DNA-containing solution was reduced to approximately 400µl by extraction with n-butanol and DNA was precipitated with 1ml 96% ethanol. Purified oligonucleotides were collected by centrifugation, redissolved in 20µl TE buffer and

25 quantified by spectrophotometry. The final yield of oligonucleotide was typically 5 to 30µg. Oligonucleotides 1 and 7 to 13 were synthesised and purified by Macromolecular Resources, USA.

Complementary oligonucleotide pairs (1µg each,

30 approximately 30pmol for 95-mer) were annealed in 10µl buffer containing 50mM Tris.HCl pH7.5, 10mM MgCl₂. The mixture was overlaid with 12µl paraffin oil, heated to 95°C and cooled to 16°C over 16h in a microprocessor-controlled heating block (Perkin Elmer Cetus Thermal

35 Cyclor). Annealed samples were transferred to clean microcentrifuge tubes and a small aliquot (1µl) withdrawn for analysis by agarose gel electrophoresis.

For each block comprising three complementary oligonucleotide pairs (blocks 1-3), four separate reactions were set up (A+B, A+C, B+C and A+B+C). Each reaction contained 50mM Tris.HCl, 10mM MgCl₂, 1mM ATP, 3mM DTT, 3μl each of the appropriate annealed oligonucleotide pairs, 0.5μl (0.5U) T4 DNA ligase and Milli-Q water to a total volume of 10μl. All ingredients except ATP, DTT and T4 ligase (Boehringer Mannheim, Germany) were mixed and heated to 45°C for 5 minutes to denature cohesive termini and then cooled to room temperature before addition of the remaining components. Ligation reactions were incubated overnight at 16°C and analysed on 2% LMT agarose gels, and ligated blocks were purified on preparative 2% LMT agarose gels with TAE running buffer. Product bands were identified under long-wave UV illumination with reference to known DNA size-standards (pBSIISK+ digested with either *Hae*III or *Alu*I) (Statagene, USA) and excised in the minimum possible volume of gel. DNA was recovered from LMT agarose fragments using GELase or agarase. Block 4 was constructed similarly, except that four instead of three complementary oligonucleotide pairs participated in the ligation reaction. Purity and yield of recovered SHLOX blocks was assessed by analytical agarose electrophoresis alongside known DNA standards.

pBSIISK+ (Stratagene) DNA was digested with appropriate restriction enzymes (Figure 2). Inserts were purified at each stage by preparative gel electrophoresis. Plasmid DNA was isolated from agarose gels (Prep-A-Gene, Bio-Rad). Approximately 100ng (ca. 0.05pmol) purified plasmid fragment was added to 50ng (ca. 0.3pmol) synthetic block in 17μl buffer containing 50 mM Tris.HCl pH 7.5, 10mM MgCl₂ and the solution heated at 45°C for 5 minutes to denature cohesive termini. Upon cooling to room temperature, 2μl 10mM ATP, 30mM DTT and 1μl T4 DNA ligase (1U) were added and the reaction incubated overnight at 16°C. The mixture was heated for 10 minutes at 75°C to inactivate T4 ligase, and DNA was

precipitated by adding 2.5 μ l 3M sodium acetate and 250 μ l ethanol to the 20 μ l volume of the ligation mixture. The pellet was resuspended in a final volume of 10 μ l TE, pH 8.0. Of this solution, 1 to 2 μ l was used to

5 electroporate 40 μ l XL1Blue competent cells (Bio-Rad electroporation Gene Pulser and Pulse Controller; 2500V/0.05sec). Electroporated cells were diluted with 1ml LB media immediately after the procedure, incubated 1 hour at 37°C and 100 μ l of this was spread on agar plates

10 containing 100 μ g/ml ampicillin (LB plus ampicillin), 0.1mM IPTG and 80 μ g/ml X-gal. Recombinants were selected on the basis of a white bacterial colony phenotype, although some blue colonies were used as controls. Plasmid DNA was screened by restriction digestion and

15 confirmed by sequencing. Plasmids containing the four lysyl oxidase blocks were designated pSHLOX1-4.

All candidate clones were screened and the mutants discarded, unless they contained mutations of potential value in future SHLOX studies. Plasmids bearing correct

20 sequences were grown in *E.coli* XL1Blue cells (Bullock et al 1987), purified by CsCl-ethidium bromide density gradient centrifugation and confirmed by sequencing in both directions.

In two separate reactions, adjacent pairs of gene

25 blocks 1+2 and 3+4 were ligated in pBSIISK+. These were designed to give recombinants α and β , respectively. Each block was excised from the appropriate plasmid and purified through agarose gel electrophoresis. 50ng each synthetic block and 100ng appropriately restriction

30 enzyme cleaved complementary-tailed pBSIISK+ digest were ligated in a total of 20 μ l T4 ligase buffer in the presence of 1U T4 DNA ligase overnight at 16°C. Transformants were screened by the colony white phenotype, comparative electrophoretic mobility of the

35 intact plasmids in 0.7% TBE agarose gel and by restriction digestion. After verification of the primary structure by sequencing, pSHLOX α and pSHLOX β were used for the final assembly of the pSHLOX gene.

pSHLOX α and pSHLOX β plasmids were cut and the appropriate fragments containing α and β subassemblies of the intact gene of lysyl oxidase were purified from an agarose gel and ligated. Transformants were selected from LB plus ampicillin plates containing IPTG and X-gal. Correct constructs were confirmed by sequencing. The intact 1188bp synthetic gene encoding lysyl oxidase (SHLOX) was used for all further manipulations. The construct containing full length SHLOX (Figure 2) in pBSIISK+ was designated pSHLOXB.

The construction of SHLOX is exemplary of the methods used with other genes.

Construction of derivatives (including shorter forms) and placing in various *E. coli* vectors for production

1. pGEXLOX-2T

The full length SHLOX gene was excised from pSHLOXB with *Bam*HI and purified by gel electrophoresis and GELase (Epicentre Technologies, USA). Purified fragment (100ng) was ligated with 50ng pGEX-2T (Medos Co Pty Ltd) in 20 μ l total volume of ligase buffer using 1U of T4 DNA ligase. The vector DNA had been previously linearised with *Bam*HI and treated with calf intestinal phosphatase (CIP; Boehringer Mannheim, Germany). Transformants were selected on LB+ampicillin plates, screened by restriction digestion and confirmed by DNA sequencing. The SHLOX gene cloned into pGEX-2T was designated pGEXLOX-2T.

2. pGEXLOX^{ml}

*Sal*I and *Xho*I mutations were introduced (Clontech Transformer mutagenesis kit) into pGEXLOX-2T at positions -13 to +23 and 421 to 442 of the SHLOX gene using two mutagenic primers (*Xho*I: CTG GTT CCG CGT GGA TCC CTC GAG CAG CCG CCG CGT; *Sal*I: ATG GTT GGT GTC GAC CCG TAC A) (Beckman Oligofax Service, Sydney). The 424 bp DNA fragment between the introduced *Sal*I and *Xho*I sites was excised from the 5'-terminal region of SHLOX of pGEXLOX-2T by cutting with *Sal*I and *Xho*I and the remaining

plasmid DNA was gel-purified. *Sal*I and *Xho*I compatible ends were ligated. Apart from GST, the resulting construct pGEXLOX^{ml} was designed such that following thrombin cleavage of the expressed protein it would imitate a 'mature' SHLOX sequence, encoding a 30kDa protein with similar size and primary structure to the natural extracellular form of lysyl oxidase (Cronshaw et.al., 1995). Following sequence confirmation and further transformation, expression was in *E.coli* DH5 α .
The resulting expressed protein was designated GST-SHLOX.

3. p3aL^{ml} and p12aL^{ml}

pGEXLOX^{ml} was treated with *Bam*HI to release the coding sequence SHLOX^{ml} (the truncated form of SHLOX). This was ligated, in each case, into the *Bam*HI site of pET3a and pET12a plasmid vectors (Novagen Inc, USA) and the resulting constructs, p3aL^{ml} and p12aL^{ml} were transformed into *E.coli* HMS174 (Studier et al 1990). Transformants were plated and screened. Orientation of inserts was confirmed by restriction digestion and the regions around the cloning sites by sequencing. Following further transformation, expression was in *E.coli* BL21/DE3 (Studier et al 1990) cells for p3aL^{ml} and p12aL^{ml}. The resulting expressed proteins were designated 3aL^{ml} and 12aL^{ml}, respectively.

4. p3DLox

Full length SHLOX was gel-purified from pSHLOXB after *Bam*HI digestion. pET3d vector was linearised with *Bam*HI and dephosphorylated with CIP. Ligation of 100ng treated vector and 50ng purified SHLOX gene with T4 DNA ligase was conducted in 20 μ l volume at 16°C for 20h, and the ligation mixture was used to electroporate *E.coli* HMS174. The recombinant bearing a SHLOX insert in the correct orientation was treated with *Nco*I to release the 842bp fragment consisting of 39 bp of pET3d and 813bp

SHLOX. Following gel purification, the resulting linear plasmid was recircularised and cloned in *E.coli* HMS174. Clones were sequenced. The verified construct (designated p3DLox) contained the 297bp SHLOX 3'-terminal region encoding a 13,653 Da polypeptide (designated Dlox) corresponding to a carboxyterminal sequence of human lysyl oxidase.

5. p3dL^{m2}

Sequences of both the 3aL^{m1} and the 12aL^{m1} recombinant polypeptides were not considered to precisely reflect the primary structure of mature human lysyl oxidase. Each had N-terminal extensions (of the order of 14 residues for 3aL^{m1} and 24 residues for 12aL^{m1}) originating as a consequence of cloning and derived from their vector sequences. These were foreign to mature human lysyl oxidase. To minimise the possible interference of these extensions on the properties and structure of the recombinant mature protein, it was considered worthwhile to place the translational start site as close as possible to aspartate₁₄₄ of SHLOX. Accordingly use was made of an internal methionine₁₄₁ residue fortuitously located just three residues upstream of aspartate₁₄₄ comprising the proposed N-terminus of the processed protein. Although there was no convenient recognition site in this region, and in particular no *Nco*I or *Nde*I endonuclease restriction site in this part of the SHLOX sequence, an appropriate modification was planned by site-directed mutagenesis. The plasmid chosen for mutagenesis was pGEXSHLOX. pGEXSHLOX is identical to pGEXLOX-2T. An *Nco*I primer was designed (CCC GTG TTG ACG CCA TGG TTG GTG) which generated two nucleotide changes to transform the GGT ATG GTT target sequence into GCC ATG GTT. After generating the mutation, the resulting construct had two *Nco*I sites; the second (downstream) site was already in the SHLOX sequence. This offered a simple clone construction scheme. After screening, the mutant clone was used to prepare purified plasmid by

CsCl-ethidium bromide density gradient centrifugation. From this DNA the 396 bp fragment, flanked by *NcoI* sites at positions 419 to 424 and 815 to 820 of the mutated SHLOX, was excised. This fragment was then inserted into pDLox. The orientation and primary structure of the recombinant was confirmed by sequencing. The resulting construct, designated p3dL^{m2}, was electroporated into *E. coli* BL21/DE3. The inventor recognises that the Asp 144 form can be generated by, for example, treatment of any of the pGEXLOX^{m1} or p3aL^{m1} or p12aL^{m1} forms with an appropriate protease, for example procollagen C-proteinase.

6. Yeast constructs

Various constructs were made for expression in yeast, including pCNNinv (Carlsberg Research Laboratories) and pYEX-BX (AMRAD). *Pichia* was chosen as an alternative expression host. For pCNNinv, a form of the synthetic gene corresponding to mature secreted lysyl oxidase was generated by PCR from p12aL^{m1}, using primers which included at their 5' termini *EagI* sites. The amplification product was purified from an agarose gel using agarase, ligated to pGEM-T and transformed into *E. coli* XL1-Blue cells. Nine colonies from 16 candidate clones generated DNA that upon cutting with *EagI* contained bands of approx. 750 and 3004 bp. Two colonies were selected for further investigation. Manual sequencing of one of the two recombinants showed a deletion. The other clone was correct over the sequenced region. A Qiagen (GmbH, Germany) preparation of DNA from this clone was cut with *EagI* in parallel with pCNNinv. Both forms of DNA were purified using agarase. The cut pCNNinv was treated with calf intestinal alkaline phosphatase and purified by phenol/chloroform extraction. The vector and insert were ligated and transformed into *E. coli* XL1-Blue cells. Twenty colonies were screened using *HindIII*, and two colonies appeared to have inserts in the correct orientation. Spheroplasts of

Saccharomyces cerevisiae strain DBY747 (AMRAD) were transformed separately with the two constructs, and one of these was subsequently chosen for detailed analysis. Complete DNA sequencing confirmed the correct sequence.

5 For cloning into pYEX-BX, the vector was cleaved with *Bam*HI and dephosphorylated using calf intestinal alkaline phosphatase. Separately, pGEXLOX-2T was treated with restriction enzymes *Not*I and *Bam*HI and the SHLOX fragment was purified from an agarose gel. This fragment
10 was ligated to the linearised vector, after which ends were filled using deoxynucleoside triphosphates and Sequenase v2.0 (Amersham). Blunt end ligation was then carried out. Correct constructs were identified by restriction enzyme digestion followed by complete
15 sequencing of the fragment and adjacent vector sequences.

All restriction enzymes and agarase used in this study were obtained from Boehringer Mannheim, Germany.

Preparation of various soluble proteins

20 Growth of *E. coli* cultures for recombinant lysyl oxidase preparation

A single colony from an agar plate was grown overnight with gyrotary shaking in 3ml LB + ampicillin media, and the entire culture was used to inoculate 250ml 2TY medium containing $70\mu\text{gml}^{-1}$ ampicillin in a 1L conical
25 flask. The inoculated medium was grown at 37°C with vigorous shaking (280-290rpm) until $\text{OD}_{600} \sim 0.6-0.8$ (about 2h) upon which expression was induced by the addition of IPTG (usually 0.4mM IPTG for DLox, 3aL^{ml} and $3\text{dL}^{\text{m}2}$; 0.1mM IPTG for GST-SHLOX). Prior to induction a 0.5ml aliquot
30 of the cell culture was withdrawn for analysis by SDS-PAGE and to confirm the absence of inclusion bodies by phase contrast microscopy. The induced cell culture was incubated under the same conditions for a further 2.5 to 3 hours then transferred to 4°C for 30 minutes before
35 harvesting. At this point a $4\mu\text{l}$ aliquot was taken for analysis by phase contrast microscopy for the presence of

inclusion bodies. If inclusion bodies were present, this was considered a reliable indicator of successful expression. Otherwise expression was monitored by SDS-PAGE of total bacterial protein alongside a sample collected before induction.

The culture (250 ml, 4°C) was divided into 50 ml aliquots in polypropylene tubes and cells were harvested by centrifugation (10 minutes, 5,000 rpm, 4°C). The supernatant was thoroughly removed and the wet pellets were weighed. A typical yield was 0.5 to 1g packed wet cells per 50 ml culture. Pellets were either stored at -80°C for several months, or used immediately for inclusion body purification.

Purification of inclusion bodies

Thawed pellets were thoroughly resuspended on ice in ~10 vol/weight of lysis buffer (50mM TrisHCL pH8.0, 1mM EDTA, 100mM NaCl (usually 5 to 7 ml per tube) and lysed at 4°C with 1mg/ml lysozyme (added in the form of a 10-times solution in lysis buffer) for 30 minutes with constant mixing. Mixing was achieved by continuous mechanical inversion on a vertical platform at 30 to 40 rpm. To inhibit putative subsequent protease activity PMSF was then added to 0.5 mM. Quantitative cell disruption was achieved by incubation with 1% Triton-X100 (4°C, constant mixing, 30 minutes). Cell lysates showed high viscosity because of released bacterial chromosomal DNA. To reduce this effect DNA was digested until visual loss of viscosity with 0.1mg/ml DNase in the presence of 10mM MgCl₂ with constant mixing (typically 20 to 30 minutes at 4°C). Viscosity was further reduced by sonication using three bursts of 10 seconds each in tubes immersed in ice. The extent of lysis was checked by phase contrast microscopy and if necessary the lysis time was extended and/or further sonication applied.

After centrifugation (typically 5,000 rpm, 0° to 4°C, 20 minutes) the supernatant was carefully decanted and residual drops of supernatant were removed with a tissue, taking care not to disturb the pellet. Ice-cold lysis buffer (volume equal to that of the the discarded supernatant) was mixed with the pellet containing inclusion bodies, and the pellet was dispersed either by repeated passage through a blue Gilson Pipetman or by 5 to 6 seconds sonication with a microtip at nearly maximal setting.

A second centrifugation was similarly performed and the supernatant was discarded. The washed pellet was resuspended in an equal volume of ice-cold 2M urea in 10mM potassium phosphate buffer, pH 8.2, and incubated for 10 minutes with constant mixing at 4°C, followed by centrifugation at 5,000 rpm, 4°C, 20 minutes to collect purified inclusion body fraction in the separated pellet.

This last step usually extracted only a minor fraction of protein from inclusion bodies as judged by SDS-PAGE. The pellet was kept cool until solubilisation, and never frozen. Storage of the inclusion body fraction was
5 (unless frozen) in the presence of 6M urea at 4°C.

Solubilisation of inclusion body protein

Solubilisation was achieved immediately after purification using ice-cold U6KP (6M urea in 10 mM potassium phosphate buffer, pH 8.2). The solution was
10 autoclaved and stored at room temperature prior to use. The amount of U6KP used for solubilisation determined the eventual concentration of recombinant lysyl oxidase. Typically 5 ml U6KP was used to solubilise the pellet derived from 50 ml bacterial culture, to yield 2 to
15 3mg/ml total protein. Mercaptoethanol was added to 2 to 3 mM. To help solubilisation mechanical disruption with a plastic Gilson tip was initially tried but this often led to loss of material which adhered to the inside and outside of the tip. Instead, the mixture was usually
20 mechanically mixed by continuous mechanical inversion on a vertical platform at 30 to 40 rpm overnight, or until there was no sign of clumps. At all times the temperature was kept 0° to 4°C.

The total protein concentration was estimated by
25 Bradford assay, and CuCl_2 was added at 5 to 10 times molar excess over estimated protein. This was usually 200 μM CuCl_2 . Solubilised protein stocks were used directly for protein analysis or for refolding of the protein by dialysis in various buffers, including
30 phosphate buffers, depending on the nature of the experiment.

Refolding of inclusion bodies by dialysis against phosphate buffer

For dialysis against phosphate buffer 10 mM and 32
35 mM dialysis tubing (Selby, Australia) was usually used. Cut tubing was boiled in MilliQ water for 10 minutes and

briefly soaked in dialysis buffer prior to use. 10mM or 16 mM phosphate buffer was prepared from 1M KH_2PO_4 and 1M K_2HPO_4 solution stocks and chemical grade distilled water, with the occasionally pH adjusted with 1M KOH.

- 5 Dialysis of 2 or 3 ml of a 6M urea solution of recombinant protein (from 2 or 3 tubes, respectively) was routinely performed against 2 L urea-free buffer, with buffer changes after each dialysis. Copper-containing samples were first dialysed against buffer containing an
- 10 approximately equimolar concentration of CuCl_2 (usually 10-50 μM), in the expectation that removal of urea would be accompanied by integration of copper and bias towards a conformationally relevant form of the protein. Excess of copper was then removed in the following dialysis.
- 15 After two consecutive dialyses (one against a copper-containing buffer, and the other against a copper-free buffer at pH 9.6) the dialysed material was transferred into polypropylene tubes on ice. Soluble and insoluble fractions were separated by centrifugation and
- 20 analysed by SDS-PAGE. The pellet and the supernatant obtained after dialysis was stored at 4°C without obvious protein degradation for at least 3 weeks, but some solubility changes were noticed during storage.

Protein from *S. cerevisiae* cultures

- 25 To obtain lysyl oxidase in yeast cell pellets and lysates, a colony was grown in CSM-leu for 48 hours. To purify secreted protein, cells were separated from growth media by centrifugation, and the supernatant was dialysed against ice-cold U6KP. Cells were washed in 5mM KPB
- 30 (retained) then resuspended in 5mM KPB plus lyticase. After incubation for 1 hour at 30°C, cells were sonicated until apparent lysis as detected by phase contrast microscopy. The pellet and lysate were separated by centrifugation. The pellet and lysates were washed with
- 35 U6KP and dialysed separately against ice-cold U6KP then against KPB, pH 9.6.

PROTEIN STUDIES

- To confirm the identity of the expressed protein, in each case DNA sequences were determined. Purified soluble recombinant lysyl oxidase forms were all examined by
- 5 SDS-PAGE, electrospray mass spectrometry, amino acid analysis and N-terminal protein sequencing to confirm the identity of the overexpressed protein. Detailed analyses of full-length protein demonstrated the following:
- (1) the protein bound copper at ~ 1 mole Cu^{2+} per mole
- 10 protein; this assay was performed by atomic absorption spectroscopy on a Varian SpectrAA-10. Protein samples were examined for protein content using a Bradford assay at 595 nm, and the ratio molar content of copper to protein was determined.
- (2) the protein exists as multimers in solution; multimer formation was observed by column chromatography in experiments using either Sephadex G100 or G200, compared with protein standards. Aliquots were examined
- 15 spectrophotometrically at 280nm. Light scattering studies (Malvern Instruments S4700 version PCS v 1.26) showed size distributions, including approximately spherical particles at 30 to 56 nm.
- (3) multiple peak formation by ion-exchange chromatography; multiple peaks were observed for 3dLm2
- 20 when analysed on a 100 nm x 4.6 mm HperD Q column on a BioSys2000 system (Beckman, USA), using a linear NaCl gradient from) to 0.4 M NaCl, over 9 min in the presence of 6M urea and 16 mM potassium phosphate buffer, at 280nm.
- (4) organic cofactor formation as evidenced by
- 30 redox-cycling (Paz et al., 1991), covalent radiolabel attachment of ^{14}C -ethylenediamine, and Raman spectroscopy of the p-nitrophenylhydrazone
- (5) enzyme activity including conversion of recombinant
- 35 tropoelastin to aqueous-insoluble material.

In the yeast expression product studied characteristics 2 and 5 have been verified indicating that functional protein is being produced while all 5

characteristics have been verified for *E.coli* expression particularly with respect to the p3dL^{m2} construct.

Levels of expression obtained have been approximately 30% of total cell protein in *E. coli* and
5 10% of total cell protein in yeast.

Benzylamine substrate assay

The assay was performed according to the method of Trackman et al. 1979. The assay monitors at 250nm the production of benzylaldehyde from benzylamine in
10 potassium phosphate buffer at 37°C. Lysyl oxidase activity is measured spectrophotometrically at 37°C by the conversion of benzylamine to benzaldehyde at 250nm.

Three samples containing lysyl oxidase and benzylamine showed a slight increase in absorbance at 250nm. In this
15 assay the lysyl oxidase was derived from the plasmid p3dL^{m2}. Controls, including boiled lysyl oxidase and benzylamine, benzylamine and dialysis buffer (no enzyme) and lysyl oxidase and KPB (no substrate) showed no increase in absorbance at 250nm.

20 Averaging the change in absorbance at 250nm of the three lysyl oxidase and benzylamine samples and estimating the concentration of lysyl oxidase in each assay the specific activity of the enzyme was calculated at 0.0145 umol/min/mg. Wang et al. reports a specific activity of
25 0.019 U/mg.

Tropoelastin substrate assay

Tropoelastin resuspended in either KPB (10mM, pH8) or PBS (10mM PB 150mM NaCl) was mixed at a final concentration of 10mg/mL, with lysyl oxidase and placed at 37°C for 2
30 hrs. In this assay, the lysyl oxidase was derived from the p3dL^{m2} plasmid. Controls included tropoelastin + lysyl oxidase + BAPN, tropoelastin + boiled lysyl oxidase, tropoelastin + dialysis buffer, tropoelastin + buffer.

Equal amounts from each reaction were run on an 8% SDS-PAGE. On staining with Coomassie less intense tropoelastin bands were seen in the tropoelastin in KPB + LO and tropoelastin in KPB + lysyl oxidase + BAPN sample lanes compared to other lanes. This may be indicative of the presence of crosslinked tropoelastin which is too large to enter the gel. Whilst BAPN should inhibit lysyl oxidase activity, preincubation of the enzyme with BAPN for at least 2 hours at 37°C may be required prior to assaying in order for the inhibition to be effective.

The above 2 samples and the tropoelastin + buffer control were centrifuged, the supernatant removed and the precipitate sent for amino acid analysis. The 2 samples showed a decrease in percentage of total lysine compared to the control. Tropoelastin + buffer contained 6% lysine, tropoelastin + lysyl oxidase 2.5% lysine and tropoelastin + lysyl oxidase + BAPN 2.2% lysine. This is consistent with the removal of lysine residues to form cross-links such as desmosines. Bedell- Hogan et al reports the presence of 5.6% lysine residues in recombinant tropoelastin compared to 2.4% in alkali insoluble recombinant tropoelastin.

In summary, the results show a lowering of the percentage of lysine in tropoelastin, as determined by the amino acid analysis. Furthermore, an insoluble protein was produced, consistent with the production of an elastin or elastin-like product.

Tritiated tropoelastin assay

Recombinant tropoelastin was prepared essentially as described Martin et al. 1995, except that BL21 cells containing pSHELF (WO 94/14958) were grown in "complete synthetic medium without lysine" (CSM-Lys), and supplemented with L-[4,5-tritium] lysine monochloride (Amersham) at 83 Ci/mmol. Accordingly, the resulting tropoelastin was radiolabelled to approximately 40,000,000 cpm/mg protein. This was lyophilised, then redissolved in assay buffer (0.1M Na₂B₄O₇, 0.15M NaCl,

pH8) and centrifuged through Millipore Ultrafree MC for 1 hour at 7500 rpm at 4°C. The retentate was used for subsequent assays. Assays were then performed by incubation with lysyl oxidase, and assessed according to the protocol of Shackelton and Hulmes, 1990. In this assay, the lysyl oxidase was derived from the p3dL^{m2} plasmid. Tritiated tropoelastin was mixed with lysyl oxidase and left at 37°C for 16 hours. Tritiated water formed during the incubation, an indication of lysyl oxidase activity, was isolated by ultrafiltration and radioactivity quantified by liquid scintillation spectrometry. A control for endogenous lysyl oxidase-like activity in *E. coli* was generated by taking an *E. coli* strain containing the pET3d plasmid through the same purification procedure as that used to produce lysyl oxidase. A sample of this pET3d control was also mixed with tritiated tropoelastin. Preliminary results are consistent with some lysyl oxidase-like activity. In all cases samples containing lysyl oxidase produced more tritiated water than those containing pET3d (Figure 5).

INDUSTRIAL APPLICABILITY

The genetic constructs of the present invention are useful in the preparation of high yields of biocompatible biospecific lysyl oxidase useful *in vivo* applications and other situations where catalysis of amine oxidation is required. The expression products of the invention are useful as agents for crosslinking of (but not confined to) tropoelastin and collagen both *in vitro* and *in vivo*, to join primary-amine materials e.g. in surgical or veterinary applications including natural and artificial materials, regulating cell growth, interfering with/promoting fibrosis, oxidising accessible lysines (and other amines) to modify behaviour of those molecular components and as components of assay systems designed to detect/quantify amines. Further, the genetic constructs and expression products of the invention have potential in cancer therapy.

REFERENCES

- Bedell-Hogan, D., Tackman, P., Abrams, W., Rosenbloom, J. and Kagan H. (1993) *J. Biol. Chem.* **268**, 10345-10350.
- 5 Boyd, C.D., Mariani, T.J., Kim, Y. and Csiszar, K. (1995)
The size heterogeneity of human lysyl oxidase mRNA is due
to alternate polyadenylation site and not alternate exon
usage. *Mol. Biol. Reports.* 21, 95-103.
- Bullock W.O., Fernandez J.M. and Short J.M. (1987)
Biotechniques 5, 376-379
- 10 Contente, S., Kenyon, K., Rimoldi, D. and Friedman, R.M.
(1990) Expression of gene rrg is associated with
reversion of NIH 3T3 transformed by LTR-c-H-ras. *Science.*
249, 796-798.
- 15 Contente, S., Csiszar, K., Kenyon, K. and Friedman, R.M.
(1993) Structure of the mouse lysyl oxidase gene.
Genomics. 16, 395-400.
- 20 Cronshaw, A.D., MacBeath, J.R., Shackleton, D.R.,
Collins, J.F., Fothergill-Gilmore, L.A. and Hulmes, D.J.
(1993) TRAMP (tyrosine rich acidic matrix protein), a
protein that co-purifies with lysyl oxidase from porcine
skin. Identification of TRAMP as the dermatan sulphate
proteoglycan-associated 22K extracellular matrix protein.
Matrix. 13, 255-266.
- 25 Cronshaw, A.D., Fothergill-Gilmore, L.A. and Hulmes, D.J.
(1995) The proteolytic processing site of the precursor
of lysyl oxidase. *Biochem. J.* 306, 279-284.
- 30 Dimaculangan, D.D., Chawla, A., Boak, A., Kagan, H.M. and
Lazar, M.A. (1994) Retinoic acid prevents downregulation
of ras recision gene/lysyl oxidase early in adipocyte
differentiation. *Differentiation.* 58, 47-52.

- Gacheru, S.N., Trackman, P.C., Shah, M.A., O'Gara, C.Y., Spacciapoli, P., Greenaway, F.T. and Kagan, H.M. (1990) Structural and catalytic properties of copper in lysyl oxidase. *J. Biol. Chem.* 265, 19022-19027.
- 5 Gough, J.A. and Murray, N.E. (1983) Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* 166, 1-19.
- Green, R.S., Lieb, M.E., Weintraub, A.S., Gacheru, S.N., Rosenfield, C.L., Shah, S., Kagan, H.M. and Taubman, M.B.
10 (1995) Identification of lysyl oxidase and other platelet-derived growth factor-inducible genes in vascular smooth muscle cells by differential screening. *Lab. Invest.* 73, 476-482.
- Hamalainen, E.R., Jones, T.A., Sheer, D., Taskinen, K.,
15 Pihlajaniemi, T. and Kivirikko, K.I. (1991) Molecular cloning of human lysyl oxidase and assignment of the gene to chromosome 5q23.3-31.2. *Genomics.* 11, 508-516.
- Hamalainen, E.R., Kempainen, R., Pihlajaniemi, T. and Kivirikko, K.I. (1993) Structure of the human lysyl
20 oxidase gene. *Genomics* 17, 544-548.
- Kagan, H. M., (1994) Lysyl Oxidase: Mechanism, Regulation and Relationship to Liver Fibrosis. *Path. Res. Pract.* 190, 910-919.
- Kagan, H.M. and Cai, P. (1995) Isolation of active site
25 peptides of lysyl oxidase. *Meth. Enzymol.* 258, 122-132.
- Kagan, H.M., Reddy, V.B., Narasimhan, N. and Csiszar, K. (1995a) Catalytic properties and structural components of lysyl oxidase. *Ciba Foundation Symposium.* 192, 100-115; discussion 115-121.

- Kagan H.M., Reddy, V.B., Panchenko, M.V., Nagan, N., Boak A.M., Gacheru, S.N. and Thomas, K.M. (1995b) Expression of lysyl oxidase from cDNA constructs in mammalian cells: the propeptide region is not essential to the folding and secretion of functional enzyme. *J. Cell. Biochem.* 59, 329-338.
- Kagan, H.M. and Trackman, P.C. (1991) Properties and function of lysyl oxidase. *Amer. J. Resp. Cell Mol. Biol.* 5, 206-210.
- 10 Kane, J.F., Violand, B.N., Curran, D.F., Staten, N.R., Duffin, K.L. and Bogosian, G. (1992) Novel in-frame two codon translational hop during synthesis of bovine placental lactogen in a recombinant strain of *Escherichia coli*. *Nucl. Acids. Res.* 20, 6707-6712.
- 15 Kenyon, K., Contente, S., Trackman, P.C., Tang, J., Kagan, H.M. and Friedman, R.M. (1991) Lysyl oxidase and rrg messenger RNA. *Science.* 253, 802.
- 20 Kenyon, K., Modi, W.S., Contente, S. and Friedman, R.M. (1993) A novel human cDNA with a predicted protein similar to lysyl oxidase maps to chromosome 15q24-q25. *J. Biol. Chem.* 268, 18435-18437.
- 25 Kim, Y., Boyd, C.D. and Csiszar, K. (1995) A new gene with sequence and structural similarity to the gene encoding human lysyl oxidase. *J. Biol. Chem.* 270, 7176-7182.
- 30 Krzyzosiak, W.J., Shindo-Okada, N., Teshima, H., Nakajima K. and Nishimura, S. (1992) Isolation of genes specifically expressed in flat revertant cells derived from activated ras-transformed NIH 3T3 cells by treatment with azatyrosine. *Proc. Natl. Acad. Sci.* 89, 4879-4883.

Lipman, D.J. and Pearson, W.R. (1985) Rapid and sensitive protein similarity searches. *Science* 227, 1435-1441.

5 Mariani, T.J., Trackman, P.C., Kagan, H.M., Eddy, R.L., Shows, T.B., Boyd, C.D. and Deak, SB. (1992) The complete derived amino acid sequence of human lysyl oxidase and assignment of the gene to chromosome 5 (extensive sequence homology with the murine ras recision gene). *Matrix*. 12, 242-248.

10 Martin, S.L., Vrhovski, B. and Weiss, A.S. (1995) Total synthesis and expression in *Escherichia coli* of a gene encoding human tropoelastin. *Gene* 154, 159-166.

Murray, E.E., Lotzer, J. and Eberle, M. (1989) Codon usage in plant genes. *Nucl. Acids Res.* 17, 477-498.

15 Nagan, N. and Kagan, H.M. (1994) Modulation of lysyl oxidase activity toward peptidyl lysine by vicinal dicarboxylic amino acid residues. Implications for collagen cross-linking. *J. Biol. Chem.* 269, 22366-22371.

20 Newgard, C.B., Nakano, K., Hwang, P.K. and Fletterick, R.J. (1986) Sequence analysis of the cDNA encoding human liver glycogen phosphorylase reveals tissue-specific codon usage. *Proc. Nat. Acad. Sci.* 83, 8132-8136.

25 Palcic, M.M., Scaman, C.H. and Alton, G. (1995) Stereochemistry and cofactor identity status of semicarbazide-sensitive amine oxidases. *Prog. Brain Res.* 106, 41-47.

Paz, M.A., Fluckiger, R., Boak, A., Kagan, H.M. and Gallop, P.M. (1991) Specific detection of quinoproteins by redox-cycling staining. *J. Biol. Chem.* 266, 689-692.

- Shackleton, D.R. and Hulmes, D.J. (1990) Purification of lysyl oxidase from piglet skin by selective interaction with Sephacryl S-200. *Biochem. J.* 266, 917-919.
- Shah, M.A., Trackman, P.C., Gallop, P.M. and Kagan, H.M.
5 (1993) Reaction of lysyl oxidase with trans-2-phenylcyclopropylamine. *J. Biol. Chem.* 268, 11580-11585.
- Sharp, P.M. and Li, W.H. (1987) The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. *Nucl. Acids Res.*
10 15, 1281-1295.
- Shibanuma, M., Mashimo, J., Mita A., Kuroki, T. and Nose, K. (1993) Cloning from a mouse osteoblastic cell line of a set of transforming-growth-factor-beta 1-regulated genes, one of which seems to encode a follistatin-related
15 polypeptide. *Eur. J. Biochem.* 217, 13-19.
- Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Meth. Enzymol.* 185, 60-89.
- 20 Svinarich, D.M., Twomey, T.A., Macauley, S.P., Krebs, C.J., Yang, T.P. and Krawetz, S.A. (1992) Characterization of the human lysyl oxidase gene locus. *J. Biol. Chem.* 267, 14382-14387.
- Tanizawa, K. (1995) Biogenesis of novel quinone
25 coenzymes. *J. Biochem.* 118, 671-678.
- Trackman, P.C., Pratt, A.M., Wolanski, A., Tang, S.S., Offner, G.D., Troxler, R.F. and Kagan, H.M. (1990) Cloning of rat aorta lysyl oxidase cDNA: complete codons and predicted amino acid sequence. *Biochemistry* 29, 4863-4870
30 [published erratum appears in (1991) *Biochemistry* 30, 8282].

- Williamson, P.R., Moog, R.S., Dooley, D.M. and Kagan, H.M. (1986) Evidence for pyrroloquinolinequinone as the carbonyl cofactor in lysyl oxidase by absorption and resonance Raman spectroscopy. *J. Biol. Chem.* 261, 16302-16305.
- Wu, Y., Rich, C.B., Lincecum, J., Trackman, P.C., Kagan, H.M. and Foster, J.A. (1992) Characterization and developmental expression of chick aortic lysyl oxidase. *J. Biol. Chem.* 267, 24199-24206.
- 10 Zhang, S.P., Zubay, G. and Goldman, E. (1991) Low-usage codons in *Escherichia coli*, yeast, fruit fly and primates. *Gene*. 105, 61-72.
- Kim, Y., Boyd, C. D., and Csiszar, K. (1995) A new gene with sequence and structural similarity to the gene encoding human lysyl oxidase. *J. Biol. Chem.* 270, 7176-7182.
- 15 Wang, S. X., Mure, M., Medzihradsky, K. F., Burlingame, A. L., Brown, D. E. Dooley, D. M., Smith, A. J., Kagan, H. M. and Klinman, J. P (1996) A cross linked cofactor in lysyl oxidase:redox function for amino acid side chains. *Science*. 273, 1078-1084.
- 20 Trackman, P. C. and Kagan, H. M. (1979) Nonpeptidyl amine inhibitors are substrates of lysyl oxidase. *J. Biol. Chem.* 254, 7831-7836.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: The University of Sydney, N/A N/A
- (ii) TITLE OF INVENTION: SYNTHETIC POLYNUCLEOTIDES
- (iii) NUMBER OF SEQUENCES: 30
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: GRIFFITH HACK
 - (B) STREET: LEVEL 8, 168 WALKER STREET
 - (C) CITY: NORTH SYDNEY
 - (D) STATE: NEW SOUTH WALES
 - (E) COUNTRY: AUSTRALIA
 - (F) ZIP: 2060
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: AU P01565
 - (B) FILING DATE: 09-AUG-1996
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: AU P01565
 - (B) FILING DATE: 09-AUG-1996
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Kurts, ANN D
 - (B) REGISTRATION NUMBER: N/A
 - (C) REFERENCE/DOCKET NUMBER: 24078DM:ADK
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 61 2 9957 5944
 - (B) TELEFAX: 61 2 9957 6288
 - (C) TELEX: AA 26547

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1251 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "nucleic acid"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCGCTTCG CCTGGACCGT GTCCTGCTC GGGCCTTTCG AGCTCTGCGC GCTAGTGCAC	60
TGCGCCCCCTC CCGCCGCCGG CCAACAGCAG CCCCCGCGCG AGCCGCCGGC GGCTCCGGGC	120

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GCCTGGCGCC AGCAGATCCA ATGGGAGAAC AACGGGCAGG TGTTTCAGCTT GCTGAGCCTG      180
GGCTCACAGT ACCAGCCTCA GCGCCGCCGG GACCCGGGCG CCGCCGTCCC TGGTGCAGCC      240
AACGCCTCCG CCCAGCAGCC CCGCACTCCG ATCCTGCTGA TCCGCGACAA CCGCACC GCC      300
GCGGCGCGAA CGCGGACGGC CGGCTCATCT GGAGTCACCG CTGGCCGCCC CAGGCCACC      360
GCCCGTCACT GGTTCGAAGC TGGCTACTCG ACATCTAGAG CCCGCGAACG TGGCGCCTCG      420
CGCGCGGAGA ACCAGACAGC GCCGGGAGAA GTTCCTGCGC TCAGTAACCT GCGGCCGCCC      480
AGCCGCGTGG ACGGCATGGT GGGCGACGAC CCTTACAACC CCTACAAGTA CTCTGACGAC      540
AACCCTTATT ACAACTACTA CGATACTTAT GAAAGGCCCA GACCTGGGGG CAGGTACCGG      600
CCCGGATACG GCACTGGCTA CTTCCAGTAC GGTCTCCAG ACCTGGTGGC CGACCCCTAC      660
TACATCCAGG CGTCCACGTA CGTGCAGAAG ATGTCCATGT ACAACCTGAG ATGCGCGGCG      720
GAGGAAAAC TGTGCGCCAG TACAGCATA AGGGCAGATG TCAGAGATTA TGATCACAGG      780
GTGCTGCTCA GATTTCCCA AAGAGTGAAA AACCAAGGGA CATCAGATTT CTTACCCAGC      840
CGACCAAGAT ATTCTGGGA ATGGCACAGT TGTCATCAAC ATTACCACAG TATGGATGAG      900
TTTAGCCACT ATGACCTGCT TGATGCCAAC ACCCAGAGGA GAGTGGCTGA AGGCCACAAA      960
GCAAGTTTCT GTCTTGAAGA CACATCCTGT GACTATGGCT ACCACAGGCG ATTTGCATGT     1020
ACTGCACACA CACAGGGATT GAGTCCTGGC TGTTATGATA CCTATGGTGC AGACATAGAC     1080
TGCCAGTGGA TTGATATTAC AGATGTAAAA CCTGGAAACT ATATCCTAAA GGTCAGTGTA     1140
AACCCAGCT ACCTGGTTCC TGAATCTGAC TATACCAACA ATGTTGTGCG CTGTGACATT     1200
CGCTACACAG GACATCATGC GTATGCCTCA GGCTGCACAA TTTCACCGTA T              1251

```

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 417 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Arg Phe Ala Trp Thr Val Leu Leu Leu Gly Pro Leu Gln Leu Cys
1           5           10           15
Ala Leu Val His Cys Ala Pro Pro Ala Ala Gly Gln Gln Gln Pro Pro
20           25           30
Arg Glu Pro Pro Ala Ala Pro Gly Ala Trp Arg Gln Gln Ile Gln Trp
35           40           45
Glu Asn Asn Gly Gln Val Phe Ser Leu Leu Ser Leu Gly Ser Gln Tyr
50           55           60

```

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Gln Pro Gln Arg Arg Arg Asp Pro Gly Ala Ala Val Pro Gly Ala Ala
 65 70 75 80
 Asn Ala Ser Ala Gln Gln Pro Arg Thr Pro Ile Leu Leu Ile Arg Asp
 85 90 95
 Asn Arg Thr Ala Ala Ala Arg Thr Arg Thr Ala Gly Ser Ser Gly Val
 100 105 110
 Thr Ala Gly Arg Pro Arg Pro Thr Ala Arg His Trp Phe Gln Ala Gly
 115 120 125
 Tyr Ser Thr Ser Arg Ala Arg Glu Arg Gly Ala Ser Arg Ala Glu Asn
 130 135 140
 Gln Thr Ala Pro Gly Glu Val Pro Ala Leu Ser Asn Leu Arg Pro Pro
 145 150 155 160
 Ser Arg Val Asp Gly Met Val Gly Asp Asp Pro Tyr Asn Pro Tyr Lys
 165 170 175
 Tyr Ser Asp Asp Asn Pro Tyr Tyr Asn Tyr Tyr Asp Thr Tyr Glu Arg
 180 185 190
 Pro Arg Pro Gly Gly Arg Tyr Arg Pro Gly Tyr Gly Thr Gly Tyr Phe
 195 200 205
 Gln Tyr Gly Leu Pro Asp Leu Val Ala Asp Pro Tyr Tyr Ile Gln Ala
 210 215 220
 Ser Thr Tyr Val Gln Lys Met Ser Met Tyr Asn Leu Arg Cys Ala Ala
 225 230 235 240
 Glu Glu Asn Cys Leu Ala Ser Thr Ala Tyr Arg Ala Asp Val Arg Asp
 245 250 255
 Tyr Asp His Arg Val Leu Leu Arg Phe Pro Gln Arg Val Lys Asn Gln
 260 265 270
 Gly Thr Ser Asp Phe Leu Pro Ser Arg Pro Arg Tyr Ser Trp Glu Trp
 275 280 285
 His Ser Cys His Gln His Tyr His Ser Met Asp Glu Phe Ser His Tyr
 290 295 300
 Asp Leu Leu Asp Ala Asn Thr Gln Arg Arg Val Ala Glu Gly His Lys
 305 310 315 320
 Ala Ser Phe Cys Leu Glu Asp Thr Ser Cys Asp Tyr Gly Tyr His Arg
 325 330 335
 Arg Phe Ala Cys Thr Ala His Thr Gln Gly Leu Ser Pro Gly Cys Tyr
 340 345 350
 Asp Thr Tyr Gly Ala Asp Ile Asp Cys Gln Trp Ile Asp Ile Thr Asp
 355 360 365
 Val Lys Pro Gly Asn Tyr Ile Leu Lys Val Ser Val Asn Pro Ser Tyr
 370 375 380
 Leu Val Pro Glu Ser Asp Tyr Thr Asn Asn Val Val Arg Cys Asp Ile
 385 390 395 400
 Arg Tyr Thr Gly His His Ala Tyr Ala Ser Gly Cys Thr Ile Ser Pro
 405 410 415
 Tyr

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1184 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "nucleic acid"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATCCCAGCA GCAGCCGCCG CGTGAACCGC CGGCTGCTCC GGGTGCTTGG CGTCAGCAGA	60
TCCAGTGGGA AAACAACGGT CAGGTTTCT CCCTGCTGTC CCTGGGTTCC CAGTACCAGC	120
CGCAGCGTCG TCGTGACCCG GGTGCTGCTG TTCCGGGTGC TGCTAACGCT TCCGTCAGC	180
AGCCGCGTAC CCCGATCCTG CTGATCCGTG ACAACCGTAC CGCGGCCGCT CGTACCCGTA	240
CCGCTGGTTC CTCCGGTGTT ACTGCAGGTC GTCCGCGTCC GACCGCGGC CACTGGTTCC	300
AGGCTGGTTA CTCCACCTCC CGTGCTCGTG AAGCTGGTGC TTCCCGTGCT GAAAACCAGA	360
CCGCTCCGGG TGAAGTTCCA GCGCTGTCCA ACCTGCGTCC GCCGTCCCGT GTTGACGGTA	420
TGGTTGGTGA CGACCCGTAC AACCCGTACA AGTACTCCGA CGACAACCCG TACTACAACT	480
ACTACGACAC CTACGAGCGC CCGCTCCGG GTGGTCGTTA CCGTCCGGGT TACGGTACCG	540
GTTACTTCCA GTACGGTCTG CCGGACCTGG TTGCTGACCC GTACTACATC CAGGCTTCCA	600
CCTACGTTCA GAAAATGTCC ATGTACAACC TGCGTTGCGC TGCTGAAGAA AACTGCCTGG	660
CTTCCACCGC TTACCGTGCT GACGTTGCTG ACTACGACCA CCGTGTTCTG CTGCGTTTCC	720
CGCAGCGTGT TAAAAACCAG GGCACCTCCG ACTTCCTGCC GTCCCGTCCG CGTTACTCCT	780
GGGAATGGCA CTCCTGCCAC CAGCACTACC ACTCCATGGA CGAATTCTCC CACTACGACC	840
TGCTGGACGC TAACACCCAG CGTCGTGTTG CTGAAGGTCA CAAAGCTTCC TTCTGCCTGG	900
AAGACACCTC CTGCGACTAC GGTACCACC GTCGTTTCGC TTGCACCGCT CACACCCAGG	960
GTCTGTCCCC GGGTTGCTAC GACACCTACG GTGCTGACAT CGACTGCCAG TGGATCGACA	1020
TCACCGACGT TAAACCGGGT AACTACATCC TGAAAGTTTC CGTTAACCCG TCCTACCTGG	1080
TTCCGGAATC CGACTACACC AACAACGTGG TTCGTTGCGA TATCCGTTAC ACCGGTCACC	1140
ACGCTTACGC TTCCGGTTGC ACCATCTCCC CGTACTAATG ATAG	1184

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 391 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

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(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Ser Gln Gln Gln Pro Pro Arg Glu Pro Pro Ala Ala Pro Gly Ala Trp
1          5          10          15
Arg Gln Gln Ile Gln Trp Glu Asn Asn Gly Gln Val Phe Ser Leu Leu
20          25          30
Ser Leu Gly Ser Gln Tyr Gln Pro Gln Arg Arg Arg Asp Pro Gly Ala
35          40          45
Ala Val Pro Gly Ala Ala Asn Ala Ser Ala Gln Gln Pro Arg Thr Pro
50          55          60
Ile Leu Leu Ile Arg Asp Asn Arg Thr Ala Ala Ala Arg Thr Arg Thr
65          70          75          80
Ala Gly Ser Ser Gly Val Thr Ala Gly Arg Pro Arg Pro Thr Ala Arg
85          90          95
His Trp Phe Gln Ala Gly Tyr Ser Thr Ser Arg Ala Arg Glu Ala Gly
100         105         110
Ala Ser Arg Ala Glu Asn Gln Thr Ala Pro Gly Glu Val Pro Ala Leu
115         120         125
Ser Asn Leu Arg Pro Pro Ser Arg Val Asp Gly Met Val Gly Asp Asp
130         135         140
Pro Tyr Asn Pro Tyr Lys Tyr Ser Asp Asp Asn Pro Tyr Tyr Asn Tyr
145         150         155         160
Tyr Asp Thr Tyr Glu Arg Pro Arg Pro Gly Gly Arg Tyr Arg Pro Gly
165         170         175
Tyr Gly Thr Gly Tyr Phe Gln Tyr Gly Leu Pro Asp Leu Val Ala Asp
180         185         190
Pro Tyr Tyr Ile Gln Ala Ser Thr Tyr Val Gln Lys Met Ser Met Tyr
195         200         205
Asn Leu Arg Cys Ala Ala Glu Glu Asn Cys Leu Ala Ser Thr Ala Tyr
210         215         220
Arg Ala Asp Val Arg Asp Tyr Asp His Arg Val Leu Leu Arg Phe Pro
225         230         235         240
Gln Arg Val Lys Asn Gln Gly Thr Ser Asp Phe Leu Pro Ser Arg Pro
245         250         255
Arg Tyr Ser Trp Glu Trp His Ser Cys His Gln His Tyr His Ser Met
260         265         270
Asp Glu Phe Ser His Tyr Asp Leu Leu Asp Ala Asn Thr Gln Arg Arg
275         280         285
Val Ala Glu Gly His Lys Ala Ser Phe Cys Leu Glu Asp Thr Ser Cys
290         295         300
Asp Tyr Gly Tyr His Arg Arg Phe Ala Cys Thr Ala His Thr Gln Gly
305         310         315         320

```

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(2) INFORMATION FOR SEQ ID NO:5:

- (xi) SEQUENCE DESCRIPTION: SEO ID NO:5:

(2) INFORMATION FOR SEQ ID NO:6:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

(2) INFORMATION FOR SEQ ID NO:7:

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCCGCGTAC CCCGATCCTG CTGATCCGTG ACAACCGTAC CGCGGCCGCT CGTACCCGTA	60
CCGCTGGTTC CTCCGGTGTT ACTGCA	86

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTCGTCCGC GTCCGACCGC GCGCCACTGG TTCCAGGCTG GTTACTCCAC CTCCCGTGCT	60
CGTGAAGCTG GTGCTTCCCG TGCTGAAAAC CAG	93

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 91 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACCGCTCCGG GTGAAGTTCC AGCGCTGTCC AACCTGCGTC CGCCGTCCCG TGTGACGGT	60
ATGGTTGGTG ACGACCCGTA CAACCCGTAC A	91

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 88 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGTACTCCGA CGACAACCCG TACTACAACT ACTACGACAC CTACGAGCGC CCGCGTCCGG 60
GTGGTCGTTA CCGTCCGGGT TACGGTAC 88

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 88 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGGTTACTTC CAGTACGGTC TGCCGGACCT GGTGCTGAC CCGTACTACA TCCAGGCTTC 60
CACCTACGTT CAGAAAATGT CCATGTAC 88

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 97 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AACCTGCGTT GCGTGCTGA AGAAACTGC CTGGCTTCCA CCGCTTACCG TGCTGACGTT 60
CGTGACTACG ACCACCGTGT TCTGCTGCGT TTCCCGC 97

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(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 99 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
AGCGTGTAA AAACCAGGGC ACCTCCGACT TCCTGCCGTC CCGTCCGCGT TACTCCTGGG      60
AATGGCACTC CTGCCACCAG CACTACCACT CCATGGACG                               99
```

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 99 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
AATTCTCCCA CTACGACCTG CTGGACGCTA ACACCCAGCG TCGTGTGCT GAAGGTCACA      60
AAGCTTCCTT CTGCCTGGAA GACACCTCCT GCGACTACG                               99
```

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 93 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
GTTACCACCG TCGTTTCGCT TGCACCGCTC ACACCCAGGG TCTGTCCCCG GGTGCTACG      60
```

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ACACCTACGG TGCTGACATC GACTGCCAGT GGA

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(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 93 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCGACATCAC CGACGTAAA CCGGGTAACT ACATCCTGAA AGTTTCCGTT AACCCGTCCT 60

ACCTGGTTCC GGAATCCGAC TACACCAACA ACG 93

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 77 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGGTTCGTTG CGATATCCGT TACACCGGTC ACCACGCTTA CGCTTCCGGT TGCACCATCT 60

CCCCGTACTA ATGATAG 77

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 85 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

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GAAAACCTGA CCGTTGTTTT CCCACTGGAT CTGCTGACGC CAAGCACCCG GAGCAGCCGG 60
CGGTTACACG GCGGGCTGCT GCTGG 85

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 85 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGGAAGCGTT AGCAGCACCC GGAACAGCAG CACCCGGGTC ACGACGACGC TCGGGCTGGT 60
ACTGGAACC CAGGGACAGC AGGGA 85

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 88 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTAACACCGG AGGAACCAGC GGTACGGGTA CGAGCGGCCG CGGTACGGTT GTCACGGATC 60
AGCAGGATCG GGTACGCGG CTGCTGAG 88

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 91 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTCAGCACGG GAAGCACCAG CTTACAGAGC ACGGGAGGTG GAGTAACCAG CCTGGAACCA 60
GTGGCGCGCG GTCGGACGCG GACGACCTGC A 91

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GTTGTACGGG TCGTCACCAA CCATACCGTC AACACGGGAC GCGGACGCA GGTTGGACAG 60
CGCTGGAAGT TCACCCGGAG CGGTCTGGTT 90

(2) INFORMATION FOR SEQ ID NO:23:

- (A) LENGTH: 91 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGTAACCCGG ACGGTAACGA CCACCCGGAC GCGGGCGCTC GTAGGTGTCG TAGTAGTTGT 60
AGTACGGGTT GTCGTCGGAG TACTTGTACG G 91

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 98 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCAGGTTGTA CATGGACATT TTCTGAACGT AGGTGGAAGC CTGGATGTAG TACGGGTCAG 60

CAACCAGGTC CGGCAGACCG TACTGGAAGT ACCGGTAC 98

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 97 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ACACGCTGCG GGAAACGCAG CAGAACACGG TGGTCGTAGT CACGAACGTC AGCACGGTAA 60

GCGGTGGAAG CCAGGCAGTT TTCTTCAGCA GCGCAAC 97

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 96 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AATTCGTCCA TGGAGTGGTA GTGCTGGTGG CAGGAGTGCC ATTCCCAGGA GTAACGCGGA 60

CGGGACGGCA GGAAGTCGGA GGTGCCCTGG TTTTAA 96

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 88 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GCAGGAGGTG TCTTCCAGGC AGAAGGAAGC TTTGTGACCT TCAGCAACAC GACGCTGGGT 60
GTTAGCGTCC AGCAGGTCGT AGTGGGAG 88

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 94 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGCAGTCGAT GTCAGCACCG TAGGTGTCGT AGCAACCCGG GGACAGACCC TGGGTGTGAG 60
CGGTGCAAGC GAAACGACGG TGGTAACCGT AGTC 94

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 93 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TGGTGTAGTC GGATTCCGGA ACCAGGTAGG ACGGGTTAAC GGAAACTTTC AGGATGTAGT 60
TACCCGGTTT AACGTCGGTG ATGTCGATCC ACT 93

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 87 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "synthetic oligonucleotide"
- (iii) HYPOTHETICAL: YES

- 72 -

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GATCCTATCA TTAGTACGGG GAGATGGTGC AACCGGAAGC GTAAGCGTGG TGACCGGTGT 60

AACGGATATC GCAACGAACC ACGTTGT 87

THE CLAIMS

1. A synthetic polynucleotide encoding an amino acid sequence selected from the group consisting of lysyl oxidase, lysyl oxidase-like proteins and variants thereof.

2. A synthetic polynucleotide according to claim 1 wherein the synthetic polynucleotide comprises a nucleotide sequence which is derived from a nucleotide sequence expressed in a vertebrate species including a mammalian or avian species.

3. A synthetic polynucleotide according to claim 2 wherein the synthetic polynucleotide sequence comprises a nucleotide sequence which is derived from a nucleotide sequence expressed in humans.

4. A synthetic polynucleotide according to any one of claims 1-3 wherein the synthetic polynucleotide has a CAI score of at least 0.30 in a particular host cell.

5. A synthetic polynucleotide according to claim 4 wherein the particular host cell is selected from the group consisting of *E. coli*, *Saccharomyces cerevisiae* and *Pichia*.

6. A synthetic polynucleotide comprising the nucleotide sequence shown in Figure 2 (SEQ ID NO: 3) or a fragment thereof, wherein the fragment thereof encodes an amino acid sequence which retains the ability of lysyl oxidase or lysyl oxidase-like proteins to catalyse the oxidation of primary amines.

7. A synthetic polynucleotide according to claim 6 wherein the nucleotide sequence commences at the codon encoding amino acid residue 141.

8. A synthetic polynucleotide according to claim 6 wherein the nucleotide sequence commences at the codon encoding amino acid residue 144.

9. A synthetic polynucleotide according to any one
5 of claims 6 to 8 wherein at least 50% of the nucleotide mutations indicated in Figure 2 (SEQ ID NO:3) have been introduced.

10. A synthetic polynucleotide according to any one
10 of claims 6 to 8 wherein the number of nucleotide mutations which have been introduced produce a CAI score for the synthetic polynucleotide in *E. coli* of at least 0.30.

11. A synthetic polynucleotide according to any one
15 of claims 1-6 wherein at least 50% of codons for any particular amino acid are selected and altered to reflect preferred codon usage in the particular host.

12. A synthetic polynucleotide according to any one
of claims 1-6 wherein at least 50% of codons for amino acids for which altered codons are desirable are altered.

20 13. A synthetic polynucleotide according to claim 12 wherein at least 60% of codons for amino acids for which altered codons are desirable are altered.

14. A synthetic polynucleotide according to claims
25 12 wherein at least 70% of codons for amino acids for which altered codons are desirable are altered.

15. A synthetic polynucleotide comprising an
nucleotide sequence encoding a lysyl oxidase, lysyl
oxidase like protein or variant thereof, wherein all or
part of the 5' or 3' untranslated region, and/or intronic
30 region of the synthetic polynucleotide is deleted.

16. A synthetic polynucleotide sequence encoding a lysyl oxidase, lysyl oxidase-like protein or variant thereof, wherein all or part of the region encoding the signal peptide, and/or region encoding the pro-peptide,
5 of the polynucleotide is deleted.

17. A synthetic polynucleotide sequence encoding a lysyl oxidase, lysyl oxidase-like protein or variant thereof, wherein all or part of the region encoding the signal peptide, and/or region encoding the pro-peptide,
10 of the polynucleotide is deleted and wherein all or part of the 5' or 3' untranslated region, and/or intronic region of the synthetic polynucleotide is deleted.

18. A synthetic polynucleotide comprising a nucleotide sequence encoding an amino acid sequence
15 selected from the group consisting of lysyl oxidase, lysyl oxidase-like proteins and variants thereof and a further nucleotide sequence wherein the nucleotide sequence encoding lysyl oxidase, lysyl oxidase-like protein or a variant thereof and the further nucleotide
20 sequence are chemically linked so as to encode a single open reading frame.

19. A synthetic polynucleotide according to claim 18 wherein the further nucleotide sequence is chemically linked to the 5' end of the nucleotide sequence encoding
25 lysyl oxidase, lysyl oxidase-like protein or a variant thereof.

20. A synthetic polynucleotide according to claim 18 or 14 wherein the further nucleotide sequence encodes all or part of a protein which causes the lysyl oxidase,
30 lysyl oxidase-like protein or variant thereof to be expressed in any one of the following environments including the extracellular environment, the cell surface environment, the periplasmic space or the

intracellular environment, or a combination of any one of the environments.

21. A synthetic polynucleotide according to claim 20 wherein the further nucleotide sequence encodes
5 glutathione S-transferase.

22. A recombinant DNA molecule comprising a synthetic polynucleotide of any one of claims 1 to 21.

23. A recombinant DNA molecule according to claim 22, the recombinant DNA molecule further comprising
10 vector DNA.

24. A recombinant DNA molecule according to claim 23 wherein the vector is selected from the group consisting of pBR322, pBluescript II, pGEX-2T, pTrc99A, pET series vectors, pET3d, pCNNinv, pYEX-BX and derivatives of these
15 vectors.

25. A host cell containing a recombinant DNA molecule according to any one of claims 22, 23 or 24 or a synthetic polynucleotide according to any one of claims 1 to 21.

20 26. A host cell according to claim 25 wherein the host cell is selected from the group consisting of prokaryotic cells and eukaryotic cells.

27. A host cell according to claim 26 wherein the prokaryotic cells are bacteria.

25 28. A host cell according to claim 25 wherein the host cell is selected from the group consisting of *E. coli*, *Saccharomyces cerevisiae* and *Pichia*.

29. An expression product encoded by a synthetic polynucleotide of any one of claims 1 to 21.

30. A method for producing an expression product according to claim 29, the method comprising the following steps:

- (i) providing a host cell according to any one of
5 claims 25 to 28;
- (ii) maintaining the host cell in conditions suitable for expression of the synthetic polynucleotide;
and
- (iii) isolating the expression product.

10 31. A method of cross-linking molecules that contain primary amines, wherein the molecules are associated with the cell membrane or the extracellular environment, the method comprising contacting the primary amine group with an expression product according to claim 29, under
15 conditions suitable to cross-link the molecules.

32. A method of cross-linking molecules that contain primary amines *in vitro*, the method comprising the step of contacting the primary amines with an expression product according to claim 29 under *in vitro* conditions
20 which are suitable to cross-link the molecules.

33. A method of cross-linking tropoelastin, collagen or both the method comprising the step of contacting the primary amines of collagen and and/or tropoelastin with an expression product according to claim 29 under
25 conditions which are suitable to cross-link the collagen and/or with tropoelastin.

34. A method of applying at least one cross-linkable primary amine containing molecule to a surface, wherein the surface provides primary amines available for cross-
30 linking, the method comprising exposing the surface to the at least one primary amine containing molecule and an expression product according to claim 29 under conditions to permit the expression product to cause

cross linking of the molecules to each other and to the surface.

35. The method according to any one claims 31 to 34 wherein the molecule is a peptide or polypeptide and the primary amine is associated with an amino acid side chain.

36. The method of claim 35 wherein the peptide or polypeptide comprises the amino acid sequence of tropoelastin or a fragment thereof.

37. The method of claim 35 wherein the peptide or polypeptide comprises the amino acid sequence of collagen or a fragment thereof.

38. The method according to any one of claims 31 to 34 wherein the molecule is a non-peptide molecule.

39. A method of accelerating the rate of wound healing, the method comprising the step of administering an expression product according to claim 29, to a wound under conditions which are suitable for cross-linking molecules which contain primary amines.

40. A method of accelerating the rate of wound healing according to claim 39, the method further comprising the step of administering molecules that contain primary amines to the wound under conditions which are suitable for cross-linking the molecules containing the primary amine to the wound.

41. The method according to claim 40 or 30 wherein the molecules that contain primary amines are tropoelastin molecules or derivatives thereof.

42. The method according to claim 39 or 40 wherein the molecules that contain primary amines are collagen molecules or derivatives thereof.

43. The method according to claim 39 or 40 wherein
5 the molecules that contain primary amines are collagen molecules or derivatives thereof, and tropoelastin molecules or derivatives thereof.

44. A formulation for use in accelerating the rate of wound healing, the formulation comprising an
10 expression product according to claim 29 and molecules containing primary amines, together with a pharmaceutically acceptable carrier, diluent or excipient.

45. A formulation according to claim 44 wherein the
15 molecules containing primary amines include at least one of tropoelastin or a derivative thereof and collagen or a derivative thereof, together with a pharmaceutically acceptable carrier, diluent or excipient.

46. A kit for use in accelerating wound healing, the
20 kit comprising a first vial containing an expression product according to claim 29 and a second vial containing molecules which contain primary amines.

47. A kit according to claim 46 wherein the second vial contains tropoelastin or a derivative thereof.

48. A kit according to claim 46 wherein the second
25 vial contains collagen or a derivative thereof.

49. A kit according to claim 47 where the kit additionally comprises a third vial containing collagen or a derivative thereof.

50. A kit according to claim 46 wherein the second vial contains collagen or a derivative thereof and tropoelastin or a derivative thereof.

5 51. A method according to claim 39 or 40 wherein the expression product is administered to the wound by direct application of the product to the wound.

52. A method according to claim 39 or 40 wherein the expression product is administered to the wound by
10 attaching the product to a matrix which is itself administered to the wound.

53. A method according to claim 39 or 40 wherein the expression product is administered to the wound by application of a pharmaceutical formulation comprising an
15 expression product according to claim 29 and a pharmaceutically acceptable carrier, excipient or diluent.

54. A method of decelerating the rate of wound healing, the method comprising the step of administering
20 antibodies which are raised against an expression product according to claim 29, to a wound under conditions which allow the antibodies to substantially inhibit the oxidation of primary amine moieties.

55. A reagent or formulation thereof for use in
25 modifying the rate of wound healing, wherein the reagent is generated by oxidising a primary amine contained in a chemical species with an expression product according to claim 29 so as to link the chemical species to a heterologous surface.

30 56. A method of promoting fibrosis, the method comprising contacting an expression product according to claim 29 with a reagent which substantially enhances the specific activity of the expression product, under

conditions which are suitable for the oxidation of primary amine moieties.

57. A method of promoting fibrosis, the method comprising administering an expression product according to claim 29 to a particular site under conditions suitable for the oxidation of primary amine moieties.

58. A method of inhibiting fibrosis, the method comprising the step of administering antibodies which are raised against an expression product according to claim 29, to a wound under conditions which allow the antibodies to substantially inhibit the oxidation of primary amine moieties.

59. A method for screening agents which substantially enhance or substantially inhibit the specific activity of lysyl oxidase, or lysyl oxidase-like proteins or variants thereof, the method comprising the step of contacting the agent with the expression product of the invention and measuring the specific activity of the expression product.

60. A method for detecting a protein or non-protein substrate in a sample, the method comprising contacting the sample with an expression product according to claim 29 and detecting the oxidation of any primary amine moieties in the sample.

61. A method for quantifying the amount of a protein or non-protein substrate in a sample, wherein the number of primary amine moieties per substrate molecule is known, the method comprising contacting the sample with the expression product according to claim 29 and detecting the amount of oxidation of any primary amine moieties in the sample.

62. A method of cancer therapy in a host in need of such treatment, comprising administering a synthetic polynucleotide according to any one of claims 1 to 21, or an expression product according to claim 29, alone or as
5 part of a complex delivered extracellularly in the host.

63. A method of regulating cell growth, the method comprising contacting a cell with an expression product according to claim 29 under conditions which permit the regulation of cell growth.

10 64. A method of modifying a lysine or other primary amine residue, the method comprising oxidising the primary amine by exposure to an expression product according to claim 29.

65. A tissue glue comprising and expression product
15 according to claim 29 and a pharmaceutically acceptable carrier and/or diluent.

66. A tissue glue according to claim 65 which additionally comprises at least one primary amine molecule species.

20 67. An antibody raised to an expression product according to claim 29.

68. The antibody of claim 67 which is specific to an expression product according to claim 29.

69. A method of cross-linking molecules that contain
25 primary amines according to claim 35, wherein the method comprises providing a complex consisting cross-linked elastin and collagen molecules, and contacting the complex with an expression product according to claim 29.

70. A method of accelerating the rate of wound
30 healing, the method comprising the step of attaching the

expression product according to claim 29 to a matrix and applying the matrix to the wound.

71. The method of claim 70 wherein the matrix comprises a complex of cross-linked elastin and collagen
5 molecules.

1/1
 ATG GCG TTC GGC TGG ACC GTG CTC CTG CTC 31/11
 M R F A W T V L L L G P L Q L C A L V H
 61/21
 TGC GGC CCT CCC GGC GGC GGC CAA CAG CAG 91/31
 C A P P A A G Q Q Q CCC CCG CCG GAG CCG CCG GCG GCT CCG GGC
 121/41
 GGC TGG CCG CAG CAG ATC CAA TGG GAG AAC AAC GGG CAG GTG TTC AGC TTG CTG AGC CTG
 A W R Q Q I Q W E N N G Q V F S L L S L
 181/61
 GGC TCA CAG TAC CAG CCT CAG CCG CCG CCG GAC CCG GGC GGC GGC GTC OCT GGT GCA GGC
 G S Q Y Q P Q R R R D P G A A V P G A A
 241/81
 AAC GGC TOC GGC CAG CAG CCC CCG ACT CCG ATC CTG CTG ATC CCG GAC AAC CCG ACC GGC
 N A S A Q Q P R T P I L L I R D N R T A
 301/101
 GCG GCG CGA ACG CCG ACG GCC GGC TCA TCT GGA GTC ACC GCT GGC CCG CCC AGG CCC ACC
 A A R T R T A G S S G V T A G R P R P T
 361/121
 GGC CGT CAC TGG TTC CAA GCT GGC TAC TCG ACA TCT AGA GGC CCG GAA CGT GGC GGC TCG
 A R H W F Q A G Y S T S R A R E R G A S
 421/141
 CCG GCG GAG AAC CAG ACA GCG CCG GGA GAA GTT CCT GCG CTC AGT AAC CTG CCG CCG CCC
 R A E N Q T A P G E V P A L S N L R P P
 481/161
 AGC CCG GTG GAC GGC ATG GTG GGC GAC GAC OCT TAC AAC CCC TAC AAG TAC TCT GAC GAC
 S R V D G M V G D D P Y N P Y K Y S D D
 541/181
 AAC CCT TAT TAC AAC TAC TAC GAT ACT TAT GAA AGG CCC AGA CCT GGG GGC AGG TAC CCG
 N P Y Y N Y Y D T Y E R P R P G G R Y R
 601/201
 CCC GGA TAC GGC ACT GGC TAC TTC CAG TAC GGT CTC CCA GAC CTG GTG GGC GAC CCC TAC
 P G Y G T G Y F Q Y G L P D L V A D P Y
 661/221
 TAC ATC CAG GCG TOC ACG TAC GTG CAG AAG ATG TOC ATG TAC AAC CTG AGA TGC GCG GCG
 Y I Q A S T Y V Q K M S H Y N L R C A A
 721/241
 GAG GAA AAC TGT CTG GGC AGT ACA GCA TAC AGG GCA GAT GTC AGA GAT TAT GAT CAC AGG
 E E N C L A S T A Y R A D V R D Y D H R
 781/261
 GTG CTG CTC AGA TTT CCC CAA AGA GTG AAA AAC CAA GGG ACA TCA GAT TTC TTA CCC AGC
 V L L R F P Q R V K N Q G T S D F L P S
 841/281
 CGA CCA AGA TAT TOC TGG GAA TGG CAC AGT TGT CAT CAA CAT TAC CAC AGT ATG GAT GAG
 R P R Y S W E W H S C H Q H Y H S H D E
 901/301
 TTT AGC CAC TAT GAC CTG CTT GAT GCC AAC ACC CAG AGG AGA GTG GCT GAA GGC CAC AAA
 F S H Y D L L D A N T Q R R V A E G H K

Fig. 1(1)

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961/321
GCA AGT TTC TGT CTT GAA GAC ACA TCC TGT GAC TAT GGC TAC CAC AGG CGA TTT GCA TGT
A S F C L E D T S C D Y G Y H R R F A C
1021/341
ACT GCA CAC ACA CAG GGA TTG AGT CCT GGC TGT TAT GAT ACC TAT GGT GCA GAC ATA GAC
T A H T Q G L S P G C Y D T Y G A D I D
1081/361
TGC CAG TGG ATT GAT ATT ACA GAT GTA AAA CCT GGA AAC TAT ATC CTA AAG GTC AGT GTA
C Q W I D I T D V K P G N Y I L K V S V
1141/381
AAC CCC AGC TAC CTG GTT CCT GAA TCT GAC TAT ACC AAC AAT GTT GTG CGC TGT GAC ATT
N P S Y L V P E S D Y T N N V V R C D I
1201/401
CGC TAC ACA GGA CAT CAT GCG TAT GCC TCA GGC TGC ACA ATT TCA CCG TAT
R Y T G H H A Y A S G C T I S P Y

991/331
1051/351
1111/371
1171/391
1231/411

Fig. 1(2)

BamHI

2/

31/11

GA TOC CAG CAG CAG CCG CCG CGT GAA CCG CCG GCT GCT CCG GGT GCT TGG CGT CAG CAG
S Q Q Q P P R E P P A A P G A W R Q Q

61/21

91/31

ATC CAG TGG GAA AAC AAC GGT CAG GTT TTC TOC CTG CTG TOC CTG GGT TOC CAG TAC CAG
I Q W E N N G Q V F S L L S L G S Q Y Q

121/41

151/51

CCG CAG CGT CGT CGT GAC CCG GGT GCT GCT GTT CCG GGT GCT GCT AAC GCT TOC GCT CAG
P Q R R R D P G A A V P G A A N A S A Q

181/61

211/71

CAG CCG CGT ACC CCG ATC CTG CTG ATC CGT GAC AAC CGT ACC GCG GCG GCT CGT ACC CGT
Q P R T P I L L I R D N R T A A A R T R

PstI

241/81

271/91

ACC GCT GGT TOC TOC GGT GTT ACT GCA GGT CGT CCG CGT CCG ACC GCG CGC CAC TGG TTC
T A G S S G V T A G R P R P T A R R W F

301/101

331/111

CAG GCT GGT TAC TOC ACC TOC CGT GCT CGT GAA GCT GGT GCT TOC CGT GCT GAA AAC CAG
Q A G Y S T S R A R E A G A S R A E N Q

361/121

391/131

ACC GCT CCG GGT GAA GTT CCA GCG CTG TOC AAC CTG CGT CCG CCG TOC CGT GTT GAC GGT
T A P G E V P A L S N L R P P S R V D G

421/141

451/151

ATG GTT GGT GAC GAC CCG TAC AAC CCG TAC AAG TAC TOC GAC GAC AAC CCG TAC TAC AAC
M V G D D P Y N P Y K Y S D D N P Y Y N

KpnI

481/161

511/171

TAC TAC GAC ACC TAC GAG CGC CCG CGT CCG GGT GGT CGT TAC CGT CCG GGT TAC GGT ACC
Y Y D T Y E R P R P G G R Y R P G Y G T

541/181

571/191

CGT TAC TTC CAG TAC GGT CTG CCG GAC CTG GTT GCT GAC CCG TAC TAC ATC CAG GCT TOC
G Y F Q Y G L P D L V A D P Y Y I Q A S

Fig. 2(1)

601/201

631/211

ACC TAC GTT CAG AAA ATG TOC ATG TAC AAC CTG CGT TGC GCT GCT GAA GAA AAC TGC CTG
T Y V Q R M S M Y N L R C A A E E N C L

661/221

691/231

GCT TOC ACC GCT TAC CGT GCT GAC GTT CGT GAC TAC GAC CAC CGT GTT CTG CTG CGT TTC
A S T A Y R A D V R D Y D H R V L L R F

721/241

751/251

CCG CAG CGT GTT AAA AAC CAG GGC ACC TOC GAC TTC CTG CCG TOC CGT CCG CGT TAC TOC
P Q R V K N Q G T S D F L P S R P R Y S

EcoRI

781/261

811/271

TGG GAA TGG CAC TOC TGC CAC CAG CAC TAC CAC TOC ATG GAC GAA TTC TOC CAC TAC GAC
W E W H S C H Q H Y H S M D E F S H Y D

841/281

871/291

CTG CTG GAC GCT AAC ACC CAG CGT CGT GTT GCT GAA GGT CAC AAA GCT TOC TTC TGC CTG
L L D A N T Q R R V A E G H K A S F C L

901/301

931/311

GAA GAC ACC TOC TGC GAC TAC GGT TAC CAC CGT CGT TTC GCT TGC ACC GCT CAC ACC CAG
E D T S C D Y G Y H R R F A C T A H T Q

961/321

991/331

GGT CTG TOC CCG GGT TGC TAC GAC ACC TAC GGT GCT GAC ATC GAC TGC CAG TGG ATC GAC
G L S P G C Y D T Y G A D I D C Q W I D

1021/341

1051/351

ATC ACC GAC GTT AAA CCG GGT AAC TAC ATC CTG AAA GTT TOC GTT AAC CCG TOC TAC CTG
I T D V K P G N Y I L K V S V N P S Y L

1081/361

1111/371

GTT CCG GAA TOC GAC TAC ACC AAC AAC GTG GTT CGT TGC GAT ATC CGT TAC ACC GGT CAC
V P E S D Y T N N V V R C D I R Y T G H

BamHI

1141/381

1171/391

CAC GCT TAC GCT TOC GGT TGC ACC ATC TOC CCG TAC TAA TGA TAG
H A Y A S G C T I S P Y * * *

Fig. 2(2)

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Fig.3(1)

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BLOCK 1: Coding strandOligo 1

GATCCCAGCAGCAGCCGCCGCTGAACCGCCGGCTGCTCCGGGTGCTTGGCGTCAGCAGA-
TCCAGTGGGAAAACAACGGTCAGGTTTTCTCCCTG

(SEQ ID NO: 5)

Oligo 2

pCTGTCCCTGGGTTCCTCAGTACCAGCCGACGCTCGTCGTGACCCGGGTGCTGCTGTTCC-
GGGTGCTGCTAACGCTTCCGCTCAGC

(SEQ ID NO: 6)

Oligo 3

pAGCCGCTACCCCGATCCTGCTGATCCGTGACAACCGTACCGCGGCCGCTCGTACCCGT-
ACCGCTGGTTCCTCCGGTGTTACTGCA

(SEQ ID NO: 7)

BLOCK 2: Coding strandOligo 4

GGTCGTCCGCGTCCGACCGCGCGCCACTGGTTCCAGGCTGGTTACTCCACCTCCCGTGCT-
CGTGAAGCTGGTGCTTCCCGTGCTGAAAACCAG

(SEQ ID NO: 8)

Oligo 5

pACCGCTCCGGGTGAAGTTCCAGCGCTGTCCAACCTGCGTCCGCCGTCCCGTGTTGACGG-
TATGGTTGGTGACGACCCGTACAACCCGTACA

(SEQ ID NO: 9)

Oligo 6

pAGTACTCCGACGACAACCCGTACTACAATACTACTACGACACCTACGAGCGCCCGCTCCG-
GGTGGTCGTTACCGTCCGGGTACGGTAC

(SEQ ID NO: 10)

BLOCK 3: Coding strandOligo 7

CGGTTACTTCCAGTACGGTCTGCCGGACCTGGTTGCTGACCCGTACTACATCCAGGCTTC-
CACCTACGTTTCAAAAATGTCCATGTAC

(SEQ ID NO: 11)

Oligo 8

pAACCTGCGTTGCGCTGCTGAAGAAAAGTGCCTGGCTTCCACCGCTTACCGTGCTGACGT-
TCGTGACTACGACCACCGTGTTCTGCTGCGTTTCCCGC

(SEQ ID NO: 12)

Fig. 4(1)

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Oligo 9

pAGCGTGTTAAAAACCAGGGCACCTCCGACTTCCTGCCGTCCCGTCCGCGTTACTCCTGG-
GAATGGCACTCCTGCCACCAGCACTACCACTCCATGGACG (SEQ ID NO: 13)

BLOCK 4: Coding strandOligo 10

AATTCTCCCACTACGACCTGCTGGACGCTAACACCCAGCGTCGTGTTGCTGAAGGTCACA-
AAGCTTCCTTCTGCCTGGAAGACACCTCCTGCGACTACG (SEQ ID NO: 14)

Oligo 11

pGTTACCACCGTCGTTTCGCTTGACCCGCTCACACCCAGGGTCTGTCCCCGGGTTGCTAC-
GACACCTACGGTGCTGACATCGACTGCCAGTGGA (SEQ ID NO: 15)

Oligo 12

pTCGACATCACCGACGTTAAACCGGGTAACTACATCCTGAAAGTTTCCGTTAACCCGTCC-
TACCTGGTTCCGGAATCCGACTACACCAACAACG (SEQ ID NO: 16)

Oligo 13

PTGGTTCGTTGCGATATCCGTTACACCGGTCACCACGCTTACGCTTCCGGTTGCACCATC-
TCCCCGTACTAATGATAG (SEQ ID NO: 17)

COMPLEMENTARY OLIGONUCLEOTIDESBLOCK 1: Complementary strandComplementary to oligo 1

pGAAACCTGACCGTTGTTTTCCCACTGGATCTGCTGACGCCAAGCACCCGGAGCAGCCG-
GCGGTTACGCGGCGGCTGCTGCTGG (SEQ ID NO: 18)

Complementary to oligo 2

pCGGAAGCGTTAGCAGCACCCGGAACAGCAGCACCCGGGTCACGACGACGCTGCGGCTGG-
TACTGGGAACCCAGGGACAGCAGGGA (SEQ ID NO: 19)

Fig. 4(2)

Complementary to oligo 3

GTAACACCGGAGGAACCAGCGGTACGGGTACGAGCGGCCGCGGTACGGTTGTCACGGATC-
AGCAGGATCGGGGTACGCGGCTGCTGAG (SEQ ID NO: 20)

BLOCK 2: Complementary strandComplementary to oligo 4

pTTCAGCACGGGAAGCACCAGCTTCACGAGCACGGGAGGTGGAGTAACCAGCCTGGAACC-
AGTGGCGCGCGGTTCGGACGCGGACGACCTGCA (SEQ ID NO: 21)

Complementary to oligo 5

pGTTGTACGGGTCGTCACCAACCATACCGTCAACACGGGACGGCGGACGCAGGTTGGACA-
GCGCTGGAACCTCACCCGGAGCGGTCTGGTT (SEQ ID NO: 22)

Complementary to oligo 6

CGTAACCCGGACGGTAACGACCACCCGGACGCGGGCGCTCGTAGGTGTCGTAGTAGTTGT-
AGTACGGGTTGTCTCGTCGGAGTACTTGTACGG (SEQ ID NO: 23)

BLOCK 3: Complementary strandComplementary to oligo 7

pGCAGGTTGTACATGGACATTTTCTGAACGTAGGTGGAAGCCTGGATGTAGTACGGGTCA-
GCAACCAGGTCCGGCAGACCGTACTGGAAGTACCGGTAC (SEQ ID NO: 24)

Complementary to oligo 8

pACACGCTGCGGGAAACGCAGCAGAACACGGTGGTCGTAGTCACGAACGTCAGCACGGTA-
AGCGGTGGAAGCCAGGCAGTTTCTTCAGCAGCGCAAC (SEQ ID NO: 25)

Complementary to oligo 9

AATTCGTCCATGGAGTGGTAGTGCTGGTGGCAGGAGTGCCATTCCCAGGAGTAACGCGGA-
CGGGACGGCAGGAAGTCGGAGGTGCCCTGGTTTTTA (SEQ ID NO: 26)

Fig. 4(3)

BLOCK 4: Complementary strand**Complementary to oligo 10**

PGCAGGAGGTGTCTTCCAGGCAGAAGGAAGCTTTGTGACCTTCAGCAACACGACGCTGGG-
TGTTAGCGTCCAGCAGGTCGTAGTGGGAG (SEQ ID NO: 27)

Complementary to oligo 11

pGGCAGTCGATGTCAGCACCGTAGGTGTCGTAGCAACCCGGGGACAGACCCTGGGTGTGA-
GCGGTGCAAGCGAAACGACGGTGGTAACCGTAGTC (SEQ ID NO: 28)

Complementary to oligo 12

PTGGTGTAGTCGGATTCCGGAACCAGGTAGGACGGGTTAACGGAACTTTCAGGATGTAG-
TTACCCGGTTTAACGTCGGTGATGTCGATCCACT (SEQ ID NO: 29)

Complementary to oligo 13

GATCCTATCATTAGTACGGGGAGATGGTGCAACCGGAAGCGTAAGCGTGGTGACCGGTGT-
AACGGATATCGCAACGAACCACGTTGT (SEQ ID NO: 30)

Fig. 4(4)

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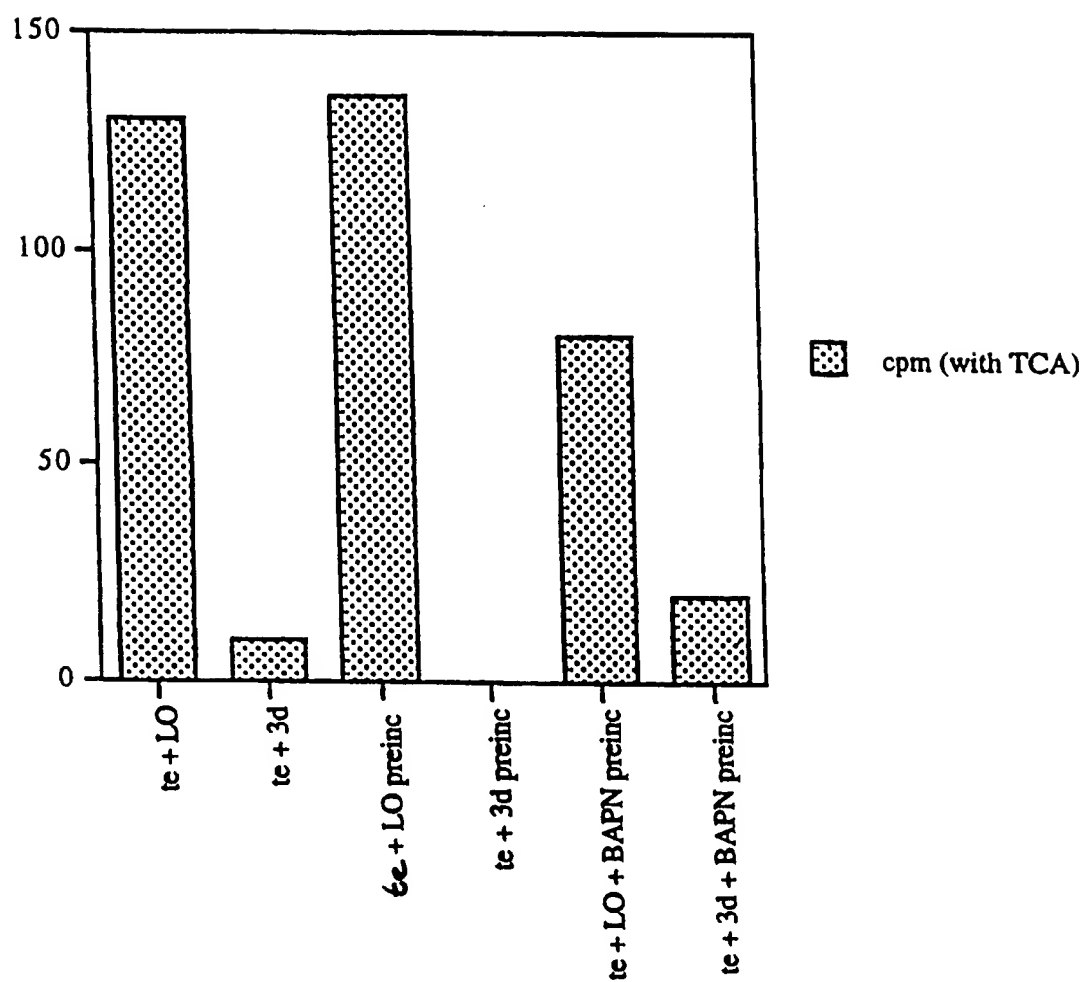


Fig. 5

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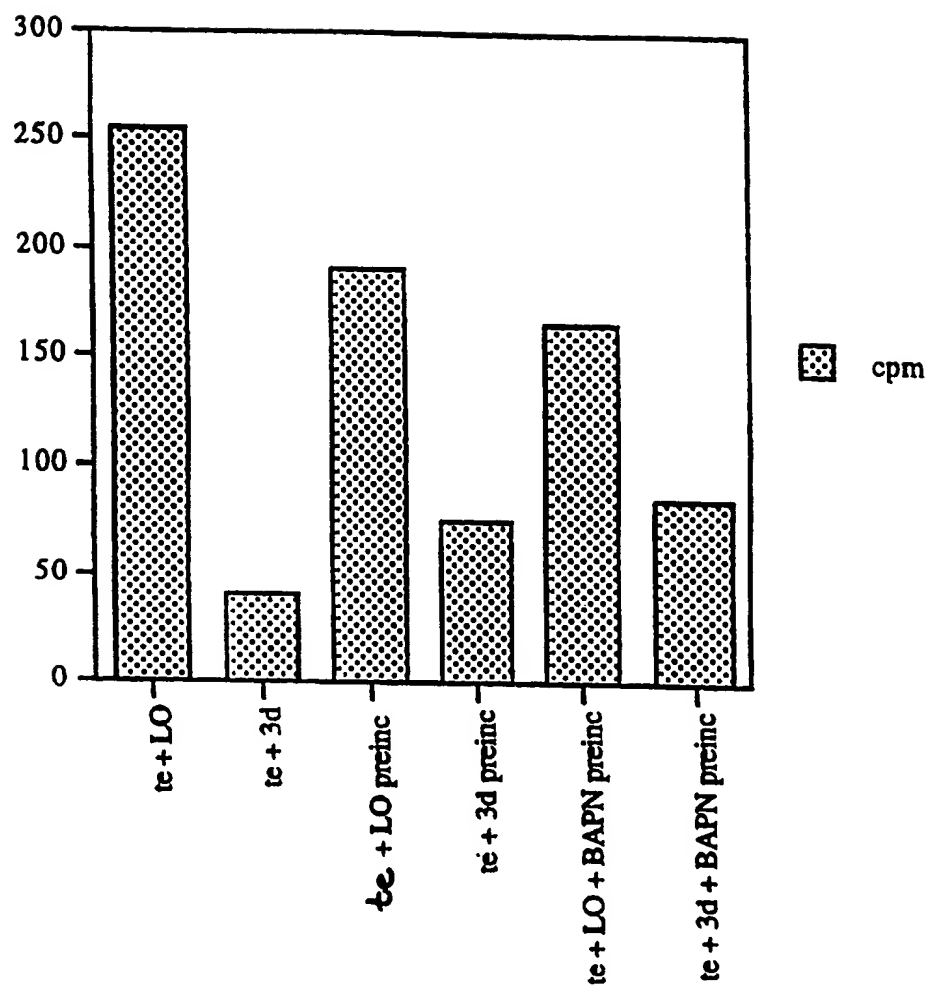


Fig. 6

INTERNATIONAL SEARCH REPORT

CORRECTED VERSION

International Application No

PCT/AU 97/00505

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁹ C12N 9/02, 15/53, A 61K 38/43.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Chemical Abstracts (CA)Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

(1) Chemical Abstracts, lysyl oxidase; Medline, lysyl oxidase /ct

(2) Genebank, EMBOL, Swiss-Prot, PIR; on SEQUENCE I.D. NO 3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	Genomics, vol. 11(3); pp 508-516, published Nov 1991 Hamalainen et al. "Molecular cloning of human lysyl oxidase and assignment of the gene to chromosome 5q 23.3-31.2"	1, 2, 3, 17, 18 19-23
X	J.Biol.chem, vol. 267 (20); pp 14382-14387, published July 15, 1992. Svinarich et al. "Characterisation of the human lysyl oxidase gene locus".	1, 2, 3, 17, 18, 19-23

☒ Further documents are listed in the continuation of Box C☐ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

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INTERNATIONAL SEARCH REPORT

International Application No.

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C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Biol. Chem, vol. 268(25), pp 18435-18437, published sept. 5, 1993. Kenyon, K et al. "A novel human CDNA with a predicted protein similar to lysyl oxidase maps to chromosome 15q24-q25".	1, 2, 3, 17, 18 19-23.
X	Genomics, vol. 17(3), pp 544-548, published sept. 1993. Hamalainen et al. "structure of the human lysyl oxidase gene".	1, 2, 3, 17, 18 19-23.
X	J. Biol. Chem, vol. 270(13), pp 7176-7182, published March 31, 1995. Kim, Y. et al. "A new gene with sequence and structure similarity to the gene encoding human lysyl oxidase".	1, 2, 3, 17, 18, 19-23
P,X	FEBS Lett, vol 399(3), pp 215-219 published Dec 16, 1996. Ouzzine, M. et al. "Expression of active, human lysyl oxidase in Escherichia coli".	1, 2, 3, 5
X	Matrix, vol. 12, pp 242-248, published 1992 Mariani, T.J. et al. "The complete derived amino acid sequence of human lysyl oxidase and assignment of the gene to chromosome 5"	
X	JP, 04-021700 (NIPPON KAYAKU) 24 Jan, 1992.	1, 2, 3, 5, 17, 18, 19, 23.